

A STUDY OF PORCINE
INTESTINAL ADENOMATOSIS

VOLUME I

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February 1978



"A Group of Difficult Organisms (G.D.O.)
(Haemophilus, Campylobacter, Elkenella, Streptobacillus)
These organisms have little in common except for the
demands they make on the skill and ingenuity of the
bacteriologist."

Cowan (1974).

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ACKNOWLEDGEMENTS

This work was carried out in the Department of Veterinary Pathology, Edinburgh University Veterinary Field Station and was supported financially by a grant from the Wellcome Trust.

I wish to extend sincere thanks to Dr. G.H.K. Lawson and Mr. A.C. Rowland of the Department of Veterinary Pathology for their constant help, advice and encouragement throughout this study.

I have pleasure in thanking the following people:

Miss P. Wooding and Mrs. N. Blair for technical advice and assistance on the bacteriological aspects of this work.

Mr. B. Kelly, Mr. K. Higginson, Mr. N. MacIntyre and Mr. M. Slater for excellent technical assistance with the pathological study.

Mr. N. Smith and Mr. I. Sneddon for assistance with and advice on electron microscopy.

Mr. R. Munro, Mr. R. James and Mr. B. Kelly for preparation of the illustrations.

Mr. D. Laing whose co-operation and assistance made the study on the closed M.D. herd possible.

Mr. I. Will for provision of experimental animals.

Mrs. H. London for help with references.

Mrs. M. Hay for assistance and advice on preparation, and Mrs. J. Wilson for typing this thesis.

SUMMARY

Field cases of porcine intestinal adenomatosis (PIA), necrotic enteritis (NE) and regional ileitis (RI) were studied. Adenomatous proliferation was present in the intestinal mucosa in all three conditions and the bacterium, Campylobacter sputorum subspecies mucosalis, was recovered consistently from the lesions. The organism was free within the apical cytoplasm of the affected intestinal epithelial cells and was not surrounded by host-cell membranes. A marked immaturity of the parasitised epithelial cells was seen but no degenerative or inflammatory changes. Whilst mucosalis isolates previously had proved remarkably homologous, a serologically distinct variant has been recovered from some cases of PIA and NE. A study of the incidence of PIA in a small closed minimal disease herd was undertaken; and also an investigation was carried out on "3 weeks scours" on a farm on which PIA was an endemic problem.

Infectivity experiments were carried out in neonatal and post-weaned piglets using cultures of mucosalis. The neonatal piglet was found to be more susceptible to infection under the conditions of the experiments. Pharmacologically-mediated inhibition of peristalsis increased the susceptibility of the post-weaned pigs to mucosalis infection. Although infections were established in both the intestinal mucosa and the oral cavity in neonatal pigs for up to six weeks, only small numbers of mucosalis were recovered and lesions of PIA were not seen.

Transmission experiments, using homogenised mucosa from animals affected with PIA, were also undertaken. PIA and NE were successfully reproduced in neonatal pigs but not in post-weaned animals.

The recovery phase of PIA was studied in both field and experimental cases. Associated with elimination of mucosalis, there was resolution of the adenomatous lesions and return of the intestinal mucosal architecture to normal. A study of cases of proliferative haemorrhagic enteropathy (PHE) showed many similarities to the recovery phase of PIA but there were also important features which possibly explain the different clinical picture seen with PHE.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Enteric diseases of the pig are of major economic importance and in recent years considerable progress towards a greater understanding of many of these has been made. Escherichia coli and its role in neonatal enteritis and post-weaning enteric disorders has received much attention especially with respect to the particular serotypes involved, the pathogenesis and the development of immunity (Moon, 1974). In swine dysentery C. coli was for many years considered to be the aetiological agent but a spirochaete, Treponema hyodysenteriae is now considered to fill that role (Taylor and Alexander, 1971; Harris *et al.* 1972). The role of other members of the enteric microbial flora in the pathogenesis of swine dysentery is not yet elucidated but progress in this field has been made (Meyer, Simon and Byerly, 1975; Alexander, Wellstead and Hudson, 1976). A highly fatal necrotic enteritis in piglets, usually less than one week old due to Clostridium perfringens type C, has been well documented (Hogh, 1967a, b; 1969a, b). Salmonella choleraesuis infection as a problem in the post-weaned pig is considered to be primarily a septicæmic disease (Lawson, 1965).

As well as these enteropathies with bacterial aetiologies a number of conditions caused by viral agents have been recognised. Transmissible gastroenteritis (TGE) due to a coronavirus (Cartwright *et al.*, 1965), and vomiting and wasting disease (VWD) of piglets due to haemagglutinating encephalomyelitis virus (HEV), another coronavirus which is distinct from the TGE virus (Phillip, Cartwright and Scott,

1971) have been described. Porcine enteroviruses may be primary enteric pathogens under certain circumstances (Dunne, 1975); and evidence has recently been provided to suggest that rotaviruses may play a role in piglet neonatal enteritis (Woode et al., 1976; and others). However, despite extensive investigations into porcine enteropathies there are still a number of conditions of which the aetiology and pathogenesis remains unknown. This is true especially of the disorders to be considered in this work: those enteropathies in which there is usually thickening of the wall of the terminal small intestine or large intestine and in which the lesion may be of a necrotic or haemorrhagic nature, but in which there is always an underlying adenomatous change in the mucosa. These conditions have been described by a plethora of terms over the years. The primary condition will be referred to as porcine intestinal adenomatosis, PIA (Rowland and Rowntree, 1972; Rowland and Lawson, 1975b). The other conditions in this complex of proliferative enteropathies will be referred to as necrotic enteritis, NE; regional ileitis, RI; and proliferative haemorrhagic enteropathy, PHE. In the past terminology for PIA and RI has included: terminal or regional ileitis (Emsbo, 1951; Field, Buntain and Jennings, 1953; Rahko and Saloniemä, 1972^a), regional enteritis (Hoorens, 1962; Nielsen, 1971), terminal jejuno-ileitis (Westendorp, 1965), intestinal adenoma (Biester and Schwarte, 1931; Moynihan and Gwatkin, 1941), proliferative ileitis (Dodd, 1968; Blood and Henderson, 1974), muscular hypertrophy with stenosis of the ileum (Nielsen, 1955; Merck Veterinary Manual, 1973).

An attempt will be made to outline our understanding of this

disease, specific aspects will then be considered in greater depth and the possible relationship of PIA to the other porcine proliferative enteropathies will be discussed.

PIA/RI

History

In 1931, Biester and Schwarte reported three cases of adenomas involving the intestines of swine. They discussed the adenomatous proliferations in the alimentary tract of other species and cited two previous cases in pigs; one of a pea to walnut sized growth composed of simple or branched gland ducts in the rectum and the other of adenomas in the large intestine. This appears to be the first time that PIA was recognised. Biester and Schwarte's cases came from a herd in which enteritis due to salmonellosis and coccidia had occurred frequently in the past. Two of the three cases showed inflammatory changes in the intestinal wall and all three showed adenomatous tissue proliferations composed of undifferentiated epithelium. They suggested that the proliferative change was primarily regenerative and developed subsequent to the initial destructive change. In 1939, Biester, Schwarte and Eveleth described a rapidly developing intestinal adenoma in a pig. The infective enteritis present in 1931 did not seem to be an added complication in this case. Moynihan and Gwatkin (1941) described a further single case of an intestinal adenoma in a pig. This was in a six-month old animal which was emaciated and had chronic diarrhoea.

Following the early reports of sporadic intestinal adenomas the first detailed study was carried out in Denmark (Emsbo, 1951). He

obtained his material from the slaughterhouse and also from autopsy cases. Emsbo divided his cases into two types: a muscular type and a mucous type. In the former muscular hypertrophy was the predominant feature, and epithelial hyperplasia in the latter; transitional forms were also described and this observation prompted Emsbo to suggest that the mucous type may be a further development of the muscular type. Emsbo's material contained cases of PIA, RI and intermediate cases, and the adenomatous hyperplasia involving the mucosa was well described but its significance was not realised. Having described the condition Emsbo stressed that it closely resembled Crohn's disease of man, a view also held by Crohn and Turner (1952).

The first description of the condition in Great Britain was in 1953 (Field, Buntain and Jennings, 1953). They recorded 17 cases of ileitis from 3 litters; 12 of these died with perforation of the affected ileum leading to peritonitis, and 5 were found on slaughter at bacon weight. Grossly in their cases the mucosa was thickened and thrown into irregular folds but histologically a subacute and chronic inflammatory reaction was described and not epithelial hyperplasia.

Muscular hypertrophy of the ileal musculature was stressed in the next report (Nielsen, 1955), in which three cases of RI with perforation and peritonitis were described. Epithelial hyperplasia was not a feature of these cases.

There was a description of adenomatous polyps of the large intestine of pigs from Hungary in 1957 (Korpassy and Tiboldi, 1957). Reference was made to earlier work published only in Hungarian (Balo and Korpassy, 1935, 1939) in which unsuccessful transmission experiments were reported.

They suggested a viral aetiology for the condition but failed to histologically demonstrate inclusion bodies in their material. Adenomatous polyps of the large intestine in pigs were reported to occur with a greater frequency in older pigs than young ones. This latter observation is in direct contrast to the findings of other workers.

In 1958, in a paper describing the establishment of pig herds free of infectious pneumonia (Pullar, 1958) several interesting observations were made on the presence of a number of enteric disorders within these herds. The ileum and caecum were examined in all pigs slaughtered as well as the lungs. During the period of the study he recorded the presence of terminal ileitis and NE in the herds. From his observation Pullar suggests that terminal ileitis "appears to be a specific disease, although to some extent both the aetiology and epidemiology are still obscure". He cites instances when pigs have died after a few days of illness, and at P.M. a NE was present involving almost all the small intestine with a complete absence of changes in the large intestine. His suggestion that these could be acute or hyperacute cases of terminal ileitis was the first mention of any possible link between the two conditions, and is of interest with respect to the recently postulated relationship between PIA, RI and NE (Rowland and Lawson, 1975b; Rowland, Lawson and Roberts, 1976).

In 1959, during transmission experiments with infectious gastroenteritis (transmissible gastroenteritis, TGE) seven cases of terminal ileitis occurred (Goodwin and Jennings, 1959). These pigs were unthrifty and at post mortem examination had changes consistent with PIA. The authors considered this evidence that PIA occurs as a chronic enteritis

following on from a number of primary infections, of which TGE is one.

Detailed studies of the condition were carried out in Belgium (Hoorens, 1962) and in Holland (Westendorp, 1965). Hoorens described two types; a muscular type and a mucous type, in agreement with Emsbo. Both workers described changes in the nerve plexuses of the bowel wall. Westendorp states that "the ganglia of Auerbach's plexus and above all of Meissner's plexus in the ileum of pigs with terminal jejuno-ileitis had enlarged and the number of nerve cells and nerve fibres had increased". Westendorp disagreed with Emsbo over the similarity to Crohn's disease of man and after studying both porcine and human material concluded that there was no pathological similarity.

Dodd (1968) described a nodular proliferation of the ileal mucosa of a young pig, for which he preferred the designation adenomatous intestinal hyperplasia. A second pig from the same litter which died of an acute necrotic ileitis was mentioned. This would appear to have been a case of NE (Rowland and Lawson, 1975b).

At a symposium on Crohn's disease held in Sweden, the main subject of a contribution on regional enteritis in domestic animals (Nielsen, 1971) centred on a discussion of the condition in the pig. It was suggested that the pig disease, although not comparable in all aspects, may be a useful model for comparative study of the human disease.

A haemorrhagic bowel syndrome followed by intestinal adenomatosis occurred in a minimal disease (MD) pig herd (Rowland and Rowntree, 1972), the whole episode lasted four months. The haemorrhagic enteropathy affected animals of all ages whilst the PIA affected only post-weaned pigs. Although no aetiological agent was identified, the cause and pathological changes suggested an infectious disease.

The pathology, occurrence, significance and histochemistry of RI of pigs in Finland has been discussed (Rahko and Saloniemi, 1972a, b, c, d; Saloniemi, et al., 1972) and acute and chronic types of the disease were described. The authors claimed that these were related to acute ileitis and Crohn's disease, respectively, in man. One of their cases which they referred to as exceptional showed an adenomatous hyperplasia of the ileum. They described inflammatory changes rather than an epithelial hyperplasia in their material.

Intracellular organisms were described in the immature proliferating epithelial cells in the affected mucosa in PIA in 1973 (Rowland, Lawson and Maxwell, 1973). The bacteria with the characteristics of a vibrio were free within the apical cytoplasm of the epithelial cells. Detailed bacteriological, immunofluorescence and electron microscopic studies were reported in 1974 (Rowland and Lawson, 1974; Lawson and Rowland, 1974). The bacterium involved was characterised and shown to be different from the previously described vibrio species isolated from pigs. The name Campylobacter sputorum subspecies mucosalis was proposed for the organism (Lawson, Rowland and Wooding, 1975). A similar host cell-bacterial relationship was described from Sweden (Martinsson et al., 1974). Further studies from Sweden included the confirmation of the presence of the organism in cases of regional ileitis (Jonsson and Martinsson, 1976) and Martinsson, Ekman and Jonsson (1976) carried out haematological and biochemical analysis of blood and serum. The first isolation of C. sputorum ss mucosalis outside the U.K. was made in Sweden (Gunnarsson et al., 1976).

Clinical Manifestations

PIA and RI are most often seen in post-weaned pigs, commonly between six and fourteen weeks of age. Cases are recorded in older animals and the case described by Moynihan and Gwatkin (1941) was in a six month old animal. The material in the studies reported by Emsbo (1951), Hoorens (1962), Westendorp (1965) and Rahko and Saloniemä (1972a) was largely derived from bacon pigs at abattoirs and therefore involved an older age group of animals. In the post-weaned pig the condition is most often recognised due to anorexia and wasting (Nielsen, 1971; Rowland and Rowntree, 1972; Rowland and Lawson, 1974; Martinsson et al., 1974; Jonsson and Martinsson, 1976). The clinical course may be more acute if there is perforation with subsequent peritonitis (Blood and Henderson, 1974; Field, Buntain and Jennings, 1953). The disease is reported as an incidental finding in bacon-weight pigs at slaughter (Emsbo, 1951; Hoorens, 1962; Nielsen, 1971; Rahko and Saloniemä, 1972a), although Emsbo states that some producers observed a lack of appetite in affected pigs. These incidental cases at slaughter would appear to indicate sub-clinical disease.

In PIA there is decreased serum albumin (Nielsen, 1967; Martinsson et al., 1974), decreased serum zinc concentration (Martinsson and Ekman, 1974), decreased total protein, transferrin and alkaline phosphatase in serum (Martinsson et al., 1976). The numbers of white blood cells, the serum cortisol and α_1 -antitrypsin levels are significantly increased (Martinsson et al., 1976).

Scouring is not a prominent feature in PIA or RI, but if necrosis of the mucosa, with subsequent development of NE has occurred, then diarrhoea is a likely sign. The diarrhoeic faeces often contain flecks

of necrotic mucous membrane. Pigs with NE become progressively more depressed, then comatose and die.

In PHE onset is rapid with the pig passing large quantities of black, foul-smelling altered blood in the faeces. Death is often sudden in PHE and indeed pigs may be found dead without having shown premonitory signs. A more chronic form, lasting several days with most pigs recovering, has been described by Pill (1971). PHE is reported most often from MD Herds (O'Neill, 1970; Rowland and Rowntree, 1972; Love, Love and Edwards, 1977). The disease is commoner in adult pigs, but Rowland and Rowntree (1972) described an outbreak in an MD herd involving all ages of pig over three weeks.

Pathology

Gross Findings

The animals are often in poor bodily condition (Biester, Schwarte, and Eveleth, 1939; Emsbo, 1951; Nielsen, 1971; Rowland and Rowntree, 1972; Rowland and Lawson, 1974; Martinsson *et al.*, 1974; Jonsson and Martinsson, 1976). There is widespread agreement that the lesions in this condition are restricted to the alimentary tract. In most descriptions the thickening of the wall of the terminal small intestine, i.e. the ileum, which may also involve the mid-small intestine or extend into the caecum and colon for a variable distance, is stressed. Cases are also seen involving only the large intestine with no abnormality in the small intestine. There is subserosal oedema and reticulation of the serosal surface with the mucosa thrown into deep folds, not unlike the lesions of Johne's disease in ruminants. These changes may be diffuse

involving the whole mucosa, but especially towards the periphery of an area of involvement isolated nodules may occur. Discrete polyps are also described, especially in the caecum and colon. Marginal cases are difficult to recognise and require histological confirmation (Rowland and Rowntree, 1972).

Two forms of the condition, a muscular type and a mucous type, have been described (Emsbo, 1951; Hoorens, 1962; Westendorp, 1965), distinction being made depending on which component of the wall is most thickened. These descriptions would seem to correspond to RI and PIA respectively and other authors believe that intermediate cases are seen (Emsbo, 1951; Rowland and Lawson, 1975b). Ulceration of the mucosa which occasionally leads on to perforation and subsequent peritonitis is described (Emsbo, 1951; Field, Buntain and Jennings, 1953; Nielsen, 1955). This is a finding in RI but not in uncomplicated PIA cases. Muscular hypertrophy with diverticulosis has been described in New Zealand (Cordes and Dewes, 1971), with herniation of the mucous membrane through the muscle coats. Hoorens (1962) also reports one such case.

To summarise, in cases of PIA the thickening of the ileum, possibly extending proximally or distally from this site, or of the large intestine alone, is due to a thickening of the mucosa with exaggerated folding. In the cases of RI conforming to the classical description "hose-pipe gut" there is a thickening of the ileum which is firm and almost rigid due to a massive hypertrophy of the ileal musculature, the submucosa being thickened with granulation tissue and the mucosa only irregularly present. Stages intermediate between these two are commonly seen.

Histopathology

Histology of normal intestine

The normal histology of the small intestine is well described in standard texts. The glandular mucosa of the small intestine consists of finger-like villi, which merge with the crypts at their bases. The villi are longer in the jejunum cf. the duodenum or ileum, and in the ileum they are shorter over the lymphoid tissues than in adjacent areas (Sloss, 1954). The Peyer's patches are a characteristic feature of the ileum and according to Titkemeyer and Calhoun (1955) each pig has about 20 in the small intestine. They are easily seen due to their prominence and vary in size with age. A single large Peyer's patch, the Peyerian gland, a band about 2 metres in length is described in the ileum by Chauveau (1872).

Two cell types are recognised on the villi; columnar absorptive cells and goblet cells, whilst lining the crypts there are undifferentiated crypt cells, goblet cells, enterochromaffin cells and Paneth cells, in some species, but probably not in the pig (Trier, 1966), although they have been described by Sloss (1954). The undifferentiated crypt cells divide and migrate up the villus, maturing to become absorptive and then are shed from the villous tips.

The mucosa of the large intestine does not form folds and being devoid of villi it has a smooth surface. The crypts are straight and open ~~ant~~ onto the mucosal surface, where the epithelium is continuous with the simple columnar epithelium lining the surface. Goblet cells are numerous in the large intestine. In the crypts undifferentiated crypt cells and occasional argentaffin cells are seen.

Histology of PIA and RI

The histology of the condition has been recorded by a number of workers. The hyperplasia and immaturity of the intestinal epithelium has been described (Biester and Schwarte, 1931; Biester, Schwarte and Eveleth, 1939; Emsbo, 1951; Dodd, 1968; Rowland and Rowntree, 1972; Rowland and Lawson, 1974; Jonsson and Martinsson, 1976). This has been described as a regenerative hyperplasia to infection or other damage, but, as pointed out by Rowland and Rowntree (1972), is an unusual change for a mucous membrane in response to insult. Up until recently the inflammatory component of the infected adenomatous mucosa has received greater emphasis (Emsbo, 1951; Field, Buntain and Jennings, 1953; Rahko and Saloniemi, 1972a). In uncomplicated adenomatosis cases the thickening is due to proliferation of the epithelial cells in the small intestine. The villous pattern is lost and the glands are hyperplastic, elongated and show branching. The epithelial cells are immature, mitotic figures are frequent and goblet cells are few in number or absent. Affected areas are sharply demarcated from the surrounding normal tissue. In the large intestine the width of the mucosa is increased due to the epithelial proliferation, elongation and increased branching of the glands. As in the small intestine there is a marked absence of goblet cells in affected areas. The presence of polypoid lesions arising from the mucosa, which on section appeared wider at the surface than at the base, has already been mentioned. As in the small intestine there is a clear demarcation between normal and abnormal areas.

Regional ileitis (RI) is associated with granulation tissue proliferation in the lamina propria and submucosa, whilst the mucosa is

only irregularly present and the muscle coats are substantially hypertrophied. Scattered islands of glandular mucosa remain and are lined by immature, hyperplastic epithelium. As in PIA this epithelium contains bacterial bodies identifiable as C. sputorum ss mucosalis (Rowland and Lawson, 1975b; Rowland, Lawson and Roberts, 1976). A number of features described by Emsbo (1951) such as the presence of accumulations of epithelioid cells and foreign body giant cells in the submucosa are not mentioned by most other authors. Giant cells, often of the Langhan's type, are described in the local drainage node (Emsbo, 1951), as also are metastases (Emsbo, 1951; Westendorp, 1965; Nielsen, 1971).

Rahko and Saloniemi (1972a) divided their cases, material selected from a slaughter house on the presence of thickened ileal walls, into acute and chronic forms. In the acute form oedema and hyperaemia of the lamina propria, epithelial proliferation and hypertrophy of the ileal musculature occurred. The inflammation was not transmural and epithelioid and giant cells were not seen. In the second more chronic group all layers of the ileal wall were said to be involved in the inflammatory process. The presence of epithelioid cell-like macrophages and multinuclear giant cells was described. One of their cases was said to be grossly indistinguishable from the others but the histological illustration resembles a case of adenomatous epithelial hyperplasia. Other than this latter case their illustrations show only non-specific changes and the major part of the epithelium consist of mucus-secreting cells.

Changes in the nerve ganglia of the gut wall have been described (Hoorens, 1962; Westendorp, 1965). Westendorp considers that the ganglia

of Auerbach's and Meissner's plexuses are enlarged in the affected jejunum or ileum and the number of nerve cells and nerve fibres increased.

"Epithelial downgrowth" (Emsbo, 1951) is described by a number of authors (Emsbo, 1951; Nielsen, 1971; Jonsson and Martinsson, 1976), i.e., penetration of the crypt epithelium into deeper layers of the gut wall through the muscularis mucosae. As mentioned by Emsbo this is also seen in normal pigs in the lymphoid follicles of Peyer's patches in the ileum. Together with the extension of lymphoid elements into the lamina propria, it is one of the areas of lympho-epithelial interaction, with major immunological implications.

Ultrastructure

Ultrastructure of normal intestine

Small intestine

The ultrastructure of the epithelium of the small intestine has been reviewed (Trier and Rubin, 1965; Toner, Carr and Wyburn, 1971), without reference to species, and in addition there are descriptions of the epithelial cell at various levels of the small intestine of the pig: duodenum (Staley, Jones and Corley, 1969a), jejunum (Staley, Jones and Marshall, 1968) and ileum (Moon, 1972).

The absorptive cells on the villus are columnar in shape, with basally situated nuclei. The brush border is made up of numerous microvilli, which are covered in an acidic mucopolysaccharide coat (Ito, 1965). Below the microvilli lies a band of closely packed fine filaments, the terminal web, these filaments terminate at the tight and intermediate junctions between cells. Mitochondria are seen throughout the epithelial cell cytoplasm. In the apical cytoplasm numerous lysosomes are seen.

Both smooth and rough endoplasmic reticulum is present throughout, and the Golgi apparatus in the supra-nuclear region is well developed. The nucleus is oval, with a homogenous distribution of chromatin showing slight peripheral aggregation on the inner surface of the nuclear envelope. The nuclear envelope is interrupted by conventional nuclear pores, and a compact nucleolus is usually seen. The basal surface of the absorptive cell is closely applied to the basement membrane. There are distinct specialisations of the lateral plasma membranes between adjacent absorptive cells. At the level of the upper portion of the terminal web there is a tight junction, with fusion of the plasma membranes and complete obliteration of the intercellular space for 0.1 to 0.2 μ . Below the tight junction the intermediate junction extends for 0.2 to 0.5 μ . Desmosomes are located at various levels along the remainder of the lateral plasma membrane.

The undifferentiated crypt cells are columnar to pyramidal in shape with basally situated nuclei. The free border is poorly developed, with shorter and fewer microvilli. Attachments between adjacent cells are similar to those in mature absorptive cells. In the apical cytoplasm there are many membrane-bound secretory granules. Few lysosomes are present, there are only poorly developed membrane systems but abundant free ribosomes. The mitochondria are evenly distributed throughout the cytoplasm and are larger and have more prominent dense granules than those of the absorptive cell.

Large intestine

There are relatively few significant fine structural differences between the absorptive cells of the large intestinal and small intestinal

mucosa (Toner, Carr and Wyburn, 1971). The microvilli are often thinner and rather less closely packed together than those of the small intestinal striated border. As in the small intestine, the microvilli are less well developed in the undifferentiated crypt cells. These undifferentiated crypt cells have convoluted nuclei, abundant mitochondria, free ribosomes, scattered granular endoplasmic reticulum and a compact Golgi apparatus.

Ultrastructure of PIA and RI

Studies on the ultrastructure of the affected alimentary tract have been carried out (Rowland, Lawson and Maxwell, 1973; Rowland and Lawson, 1974; Martinsson *et al.*, 1974; Johsson and Martinsson, 1976). Ultrastructurally the adenomatous epithelial cells resemble undifferentiated crypt cells. At the surface the microvilli are poorly developed, the terminal web and membrane systems of the endoplasmic reticulum in the cell are less elaborate than in the mature absorptive cells of the villus. There are numerous ribosomes giving a granular appearance to the cytoplasm, and variably sized, electron dense secretory granules are prominent towards the free border. The nucleus and mitochondria present no unusual features. In addition to the normal cellular organelles, irregularly curved bacterial bodies are seen in the affected epithelial cells. These bacterial bodies lie free within the apical cytoplasm of the cells and are not apparently bound by membranes, although a halo, a zone of clearing, may be seen around some of them. These bodies are clearly distinguishable from the other organelles in the cells. The bacteria are occasionally seen undergoing division. The bacteria are irregularly curved, surrounded by a distinct cell wall and are osmophilic.

Histochemistry

In normal pigs alkaline phosphatase activity is strongest in the epithelial cells of the apical part of the villi; the crypt cell being non-reactive (Saloniemi and Rahko, 1972; Jonsson and Martinsson, 1976). In pigs with PIA the alkaline phosphatase activity of the affected ileal epithelium is diminished or absent (Jonsson and Martinsson, 1976). Rahko and Saloniemi studied the epithelial mucosubstances in the ileal mucosa of normal pigs (1972c) and pigs with regional ileitis (1972d). No abnormalities could be observed in the main histochemical reactions of the mucosal mucins, although the amount of mucin production was altered. As has already been discussed Rahko and Saloniemi stressed the inflammatory changes in their material and described a goblet cell hyperplasia rather than an immaturity and hyperplasia of the epithelium. In acute regional ileitis they described the mucin secretion as normal or slightly elevated. In chronic regional ileitis the mucin secretion was decreased in ulcerated areas, but remained normal in adjacent non-affected areas. An increase in the mucin secretion of goblet cells in nodular hyperplastic areas was described and here mucins were also secreted by hyperplastic epithelial cells.

In PIA with its immature hyperplastic epithelium, mucus secretion would be expected to decrease and conventional histology reveals few mucus-secreting cells (Rowland and Rowntree, 1972; Rowland and Lawson, 1974). Rahko and Saloniemi (1972d) mentioned that in their case of adenomatous hyperplasia (Rahko and Saloniemi, 1972a) the intensely proliferating intestinal epithelial cells showed an absence or irregular distribution of qualitatively normal mucin.

Aetiology and Pathogenesis

Over the years many attempts have been made to experimentally reproduce PIA or RI, and to determine their aetiology. To date successful reproduction of PIA has not been reported, although claims for reproduction of RI have been made (Kalima, Saloniemä and Rahko, 1976).

As early as 1929 - 1932 Adersen (ref. to by Emsbo, 1951) attempted to transmit the condition using bacterial cultures from the intestines of cases, and minced organ material fed to pigs. Transmission was also attempted through contact. Biester and Schwarte (1931) fed intestinal contents and scrapings from the large intestine of affected pigs and an acute dysentery was produced. Their cases had an underlying infective process, and although four cases examined histologically had epithelial proliferative changes, it is difficult to evaluate their results. In 1939, Biester, Schwarte and Eveleth carried out further transmission experiments using material from an affected pig. In this instance there was no other infective condition, but the transmission was unsuccessful. Others have also attempted unsuccessfully to transmit the condition using material from affected animals (Emsbo, 1951; Korpassy and Tiboldi, 1957; Rowland and Rowntree, 1972).

Hoorens (1962) carried out a number of experiments involving the feeding of talc and silica gel, and surgical procedures such as blockage of the lymphatics, partial occlusion of the gut lumen and injection of sclerosing substances into the gut wall and lymphatics. The changes produced were those to be expected from such surgical interference and introduction of irritant foreign substances; oedema, and

connective tissue proliferation and a foreign body response. He concluded that although the primary cause of RI was still unknown, obstruction of the lymphatics of the gut wall and mesentery were important in the pathogenesis.

Workers in Finland have also suggested that lymphatic obstruction may play an important role in the pathogenesis of RI (Kalima, 1971). Granulomatous inflammation with foreign body giant cells in the gut wall was produced after experimental lymphatic obstruction using sclerosing substances (Rahko, Saloniemi and Kalima, 1973; Saloniemi, Rahko and Kalima, 1974; Kalima, Saloniemi and Rahko, 1976). The changes produced were of an inflammatory nature and epithelial changes were not described.

A familial predisposition or occurrence of the condition has been suggested (Adersen, 1932; Emsbo, 1951; Hoorens, 1962; Merck Veterinary Manual, 1973; Blood and Henderson, 1974). This seems to be based on the fact that more than one case is often seen in a single litter. An infective nature would also provide an adequate reason for such a finding. Diverticulosis and muscular hypertrophy were said to be inherited as a simple recessive character in the Berkshire pig (Bishop cited by Cordes and Dewes, 1971).

Ludvigsen (1958) suggested that RI was due to an allergic reaction in the terminal ileum of pigs after weaning. Diagnosis was confirmed by exploratory laparotomy in his cases and the pigs treated with corticosteroids. Recovery following treatment was said to be rapid. Due to the fact that uncomplicated cases of PIA will recover without treatment (Rowland and Rowntree, 1972; Rowland and Lawson, 1974) it is difficult to assess such a claim.

The nature of the lesion, a proliferation of epithelial cells resembling an adenoma, has stimulated a number of workers to consider viruses in the aetiology (Biester, Schwarte and Eveleth, 1939; Korpassy and Tiboldi, 1957). Biester, Schwarte and Eveleth (1939), as mentioned previously, were unsuccessful in their transmission studies to investigate this suggested aetiology. Similarly the work of Korpassy and Tiboldi (1957) failed to demonstrate the presence of any viral agent. Recently there has been vigorous activity in the field of study of enteric viruses in all species. Two coronaviruses are recognised as important aetiological agents in enteric disorders in the pig; transmissible gastro-enteritis (TGE) virus and vomiting and wasting disease (VWD) virus. More recently reo-like viruses or rotaviruses have been implicated in neonatal diarrhoea in piglets (Woode, et al., 1976; Rodger, Craven and Williams, 1975; Lecce, King and Mock, 1976). Bergeland et al., (1975) described the isolation of reo-like viruses from cases of proliferative ileitis and haemorrhage bowel syndrome (these two corresponding to PIA and PHE respectively). However, these viruses were not demonstrable in all their cases of PIA or PHE, and they were also demonstrable in a wide variety of other enteric disorders.

In early work on TGE in the United Kingdom cases of RI occurred (Goodwin and Jennings, 1959) in an experimental pig herd into which TGE had been introduced. The authors concluded that this was evidence that RI was a chronic enteritis, which could follow on from a number of specific primary intestinal infections, of which TGE was one. Evidence against this is the absence of TGE and VWD from Scotland, although the proliferative enteropathies are commonly seen.

Nielsen (1955) and Westendorp (1965) suggested that ileal muscular hypertrophy was the primary change and that mucosal changes were only of a secondary nature. Later workers suggested that the muscular hypertrophy and ulceration were secondary to destruction of an already adenomatous mucosa by the normal bowel flora (Rowland and Lawson, 1975b; Rowland, Lawson and Roberts, 1976) so giving rise to the classical "hose-pipe gut" recognised by most practising pig pathologists. Evidence for this was the presence of immature hyperplastic epithelium in such cases, and the demonstrable presence in the epithelium of bacteria identical to those associated with PIA.

Necrotic Enteritis (NE)

In the past there has been much confusion regarding this condition and although well recognised in most areas of the world, a multitude of different aetiologies have been suggested. Many authors consider it to be the end-stage of several diseases or disease complexes possibly both infectious and nutritional in origin (Jubb and Kennedy, 1970; Jennings, 1959). Salmonella choleraesuis has probably been suggested most commonly as the aetiological agent and even today is accepted by some authors as being involved (Jubb and Kennedy, 1970; Hungerford, 1975), despite the fact that other workers have stressed the absence of S. choleraesuis from the lesions of NE (Jennings, 1959; V.I. Service, 1959; Smith, 1959; Pay, 1970a). As early as 1937, McEwan (1937) described an outbreak of NE in a herd free of salmonellosis. Pay (1970a) pointed out important diagnostic differences between S. choleraesuis infection and NE in fattening pigs. Pantothenic acid or nicotinic acid deficiency has also been implicated in NE (Chick et al., 1938; Davis and Freeman, 1940;

Runnels, Monlux, and Monlux, 1965; Hungerford, 1975). Stockdale (1970) stated "necrotic enteritis occurs in pigs as a sequel to vibriosis, salmonellosis, deficiency, and coliform enteritis". NE as a sequel to infection with *Oesophagostomum* spp. has also been described (Stockdale, Davenport and Miniats, 1969) and swine fever virus has also been proposed as a possible initiator of the condition (Smith and Jones, 1963) despite the fact that NE was described in Europe before the introduction of swine fever (Jubb and Kennedy, 1970). To stress our confused understanding of NE the reader is referred to the account of the condition by Hungerford (1975).

Recently similarities between PIA and NE have been described (Rowland and Lawson, 1975b; Rowland, Lawson and Roberts, 1976). The site of the lesion is similar for the two conditions, the gross findings in NE are such that one could envisage the NE occurring as a result of a coagulative necrosis of the mucosa of PIA. Histologically, in the underlying surviving mucosa, there is an epithelial hyperplasia with a marked immaturity of the cells and an absence of goblet cells. An identical organism to that described in PIA can be demonstrated in these immature proliferating cells and cultured from the lesions.

Proliferative Haemorrhagic Enteropathy (PHE)

Haemorrhagic enteropathies in the pig, although of considerable importance to the pig industry world-wide, have been poorly classified, leading to much confusion in the literature. There seem to be at least three distinct conditions. Gastro-oesophageal ulceration with haemorrhage into the stomach (Hobson, 1961, 1971; McErlean, 1962) is well recognised, but its aetiology is unclear. A second type is that termed "the intestinal

haemorrhage syndrome" (Jennings, 1959; Jones, 1967; Pay, 1970a; Smith and Shanks, 1971; Uruchurtu and Ponce, 1971; Rowland and Lawson, 1973). This type is characterised by an acute clinical course, a pale carcass and a tense distended abdomen. The small intestine except the duodenum is tympanitic, thin-walled and intensely congested. The colon may also be involved in this condition. There is a suggested relationship between this type of intestinal haemorrhage syndrome and whey feeding (Dodd, Cordes and O'Hara, 1963; Todd *et al.*, 1977) but it is not observed exclusively in whey-fed pigs (Dodd *et al.*, 1963; Smith and Shanks, 1971). Torsion of the root of the mesentery leading to venous occlusion, a massive pooling of blood and anoxia of the affected intestine in a large percentage of such cases has been described (Rowland and Lawson, 1973). They reported that physical activity following feeding was often reported prior to death. A third entity is "haemorrhagic bowel syndrome" (O'Neill, 1970; Pill, 1971; Rowland and Rowntree, 1972; O'Hara, 1972; Redman Chu and Hong, 1973; Rowland and Lawson, 1975a; Kelly and Cameron, 1976). In this condition haemorrhage is from the ileal mucosa, although the bleeding points cannot usually be identified. Death may follow a short clinical course, with passing of blood in the faeces, although some pigs make a gradual recovery over several days (Pill, 1971; Rowland and Rowntree, 1972). A varying length of the terminal ileum is thickened and firm, with oedema of the associated mesentery. In a retrospective study Rowland and Lawson (1975a) noted the similarities between this condition, which they term PHE, and PIA, using material from the outbreak described by Rowland and Rowntree (1972). In PHE the underlying mucosa is thickened due to epithelial hyperplasia in which immaturity of the epithelial cells is a feature, and few goblet cells are present. Bacteria have been described free

within the apical cytoplasm of epithelial cells in affected mucosa (Rowland and Lawson, 1975a and 1975b; Rowland, Lawson and Roberts, 1976). These organisms morphologically resemble those seen in PIA, and there is positive immunofluorescence using hyperimmune serum prepared against the bacteria isolated from cases of PIA (Rowland and Lawson, 1975a). PHE with the presence of intracellular organisms of vibrio morphology has also been described in Australia (Kelly and Cameron, 1976; Love, Love and Edwards, 1977). Love et al., (1977) described the isolation of bacteria which morphologically and biochemically resembled C. sputorum ss mucosalis (Lawson, Rowland and Wooding, 1975).

Mycotoxins have been suggested as being of aetiological importance in PHE (Van Ulsen, 1971). In a retrospective study using meal from the outbreak of PHE described by O'Neill (1970) the concentration of scopoletin was measured (Patterson, Roberts and O'Neill, 1971). Experiments feeding up to one thousand times this concentration failed to produce a clinical response, and they concluded that this coumarin derivative was unlikely to be involved in the aetiology of PHE. An allergic basis for the condition has also been suggested (O'Neill, 1970; Pill, 1971; Redman Chu and Hong, 1973).

Confusion over the haemorrhagic enteropathies of the pig has been exacerbated by a failure to adequately define these pathologically different conditions. The differences of opinions on PHE was reflected in the variety of papers on haemorrhagic enteropathies at the I.P.V.S. Congress at Ames, Iowa in 1976. One communication dealt with PHE and its association with mycotoxins (Kurtz, Mirocha and Meade, 1976), whilst another dealt with the constant association of intracellular bacteria and stressed the similarity of the condition to PIA, RI and NE (Rowland, Lawson and Roberts, 1976).

From this time onwards the following terminology will be employed. PIA will refer to the condition in which there is thickening of the terminal small intestine and/or large intestine due primarily to epithelial hyperplasia. RI will be used for those cases in which the thickening is due to muscular hypertrophy and granulation tissue proliferation. It should be noted that Swedish workers refer to PIA as regional or terminal ileitis or regional enteritis; and in fact they do not stress the distinction between PIA and RI which will be made in this work. The NE described will be that originating in an already hyperplastic mucosa and not the condition described in association with vitamin deficiencies. PHE will refer to that condition in which there is haemorrhage from the mucosa of the terminal small intestine, which is thickened and shows an underlying epithelial hyperplasia; and not the "torsion-type" of haemorrhagic enteropathy.

Geographical Distribution

Proliferative enteropathies in the pig have been reported from most areas of the world: U.S.A. (Biester and Schwarte, 1931); Canada (Moynihan and Gwatkin, 1941); Mexico (Necoechea *et al.*, 1969); U.K. (Fielding, Buntain and Jennings, 1953; Rowland and Rowntree, 1972); Denmark (Emsbo, 1951); Belgium (Hoorens, 1962); Finland (Rahko and Saloniemi, 1972a); Sweden (Martinsson and Jonsson, 1974); Australia (Pullar, 1959; Kelly and Cameron, 1976); New Zealand (Cordes and Dewes, 1971), and India (Rajan, Nair and Maryamma, 1975). It appears that this important group of enteropathies in the pig is world-wide in distribution in all areas where pigs are kept.

Occurrence

Although there are some published figures suggesting their importance, the true incidence of the proliferative enteropathies in the pig is not known. The reasons for this are many, including the confusion surrounding these conditions in the past, the fact that it is only recently that the relationship between these conditions has been indicated, and the conditions defined in pathological terms. Also, PIA cannot be identified clinically in the pig with certainty and the only signs may be anorexia and a variable degree of wasting. These may go unnoticed and make assessment of its impact on the herd impossible to assess.

In Denmark Emsbo (1951), in autopsy material of 1,410 pigs found the following age distribution; sows and boars 0.5%, fattening pigs 0.8%, young pigs 3.0% (figures from Nielsen, 1971). In Belgium Hoorens (1962) cited a frequency of 0.1% in slaughter pigs and of 1.02% in autopsied pigs. Nielsen (1971) examined all the guts of bacon pigs in an abattoir on 2 days and the frequency was 0.4% on the first day and 0.2% on the second. In Finland, Saloniemi *et al.* (1972) found, in a 12 month survey, that 0.73% of slaughtered pigs had regional enteritis. These figures do not give the true occurrence of these conditions in the herds from which the pigs came. When one considers that affected pigs may recover, and any lesions present resolve, then it is easier to see how difficult it is to determine the true incidence.

In surveys of disease incidence in pigs, NE features prominently as a very significant disease, especially in post-weaned pigs (V.I. Report, 1959; Jennings, 1959; Pay, 1970a). Pay (1970b) in a study on the effect of disease on a large pig fattening enterprise found that NE accounted for 6.2% of the total mortality causing a financial loss at

that time of £2,033. If the hypothesis put forward by Rowland and Lawson (1975b) is true, that NE occurs due to secondary infection resulting in a coagulative necrosis of an underlying lesion of PIA, and since cases of PIA recover naturally, then it seems not unreasonable to suggest that the economic impact on the herd may be far greater. Pigs failing to gain weight satisfactorily for a period and therefore taking longer to reach marketable weight, must be an added economic loss in addition to deaths. Pullar (1958) in the setting up of virus-pneumonia free herds in Australia encountered NE and terminal ileitis and gave a morbidity rate of 10 - 30%, and an affected litter rate of 40 - 60% for each condition.

More dramatic in its effect is PHE (Rowland and Rowntree, 1972; Love, Love and Edwards, 1977). The outbreak of PHE described by Love et al., (1977) involved the breeding unit of a MD piggery of 600 sows and their progeny. In an initial outbreak lasting 12 weeks 318 clinical cases were seen with 156 deaths, giving a morbidity rate of about 12% of the breeding stock and the mortality rate of the clinically affected animals was 45% to 50% irrespective of age. This outbreak was terminated by introduction of medication in the feed. However, some weeks later, after medication of the feed had stopped, further clinical cases occurred; 54 clinical cases were seen and 30 died or were killed in extremis. These cases however, recurred only in young boars or gilts introduced 3 to 12 weeks previously into the breeding unit from the rearing unit, in the latter of which no evidence of the condition was seen.

Vibrio Infection in the Pig

For many years a vibrio, first isolated by Doyle (1944) from pigs affected with swine dysentery and named Vibrio coli (Doyle, 1948),

was thought to be the aetiological agent of this disease. Conflicting results have been obtained in the reproduction of swine dysentery using apparently pure cultures of V. coli (for discussion see Warner, 1965; Harris and Kinyon, 1975). Other workers have isolated similar bacteria from the alimentary tract and faeces of pigs (Roberts, 1956; Deas, 1960; Lussier, 1962, and others). The role of these organisms in the aetiology of swine dysentery is now in doubt (Taylor and Alexander, 1971; Harris et al., 1972). The majority of these bacteria are catalase positive and are only distinguishable from Vibrio fetus ss intestinalis with difficulty (Veron and Chatelain, 1973). In this they are distinct from the bacteria described from cases of PIA (Lawson and Rowland, 1974; Lawson, Rowland and Wooding, 1975), these being catalase negative. Catalase negative vibrios from diseased intestine have been described (Söderlind, 1965; Warner, 1965), however, insufficient information is given to allow retrospective identification of their strains, although their recorded characters suggest that they are not the same as C. sputorum ss mucosalis (Lawson, Rowland and Wooding, 1975). They more closely resemble C. coli.

Those organisms, which are microaerophilic vibrios, are now included in the genus Campylobacter, and not with Vibrio cholerae in the genus Vibrio (Smibert, 1974). Campylobacters are gram-negative, non-spore-forming, spirally curved rods with a single polar flagellum at one or both ends of the cell. They are motile with a characteristic corkscrew-like motion. Carbohydrates are neither fermented nor oxidised and serum is not required for growth. Energy is from amino acids or tricarboxylic acid intermediates. They are microaerophilic to anaerobic in their requirements (Smibert, 1974).

The catalase negative campylobacters are represented by a single species, Campylobacter sputorum within which there are three subspecies; subspecies sputorum, subspecies bubulus (Veron and Chatelain, 1973) and subspecies mucosalis (Lawson, Rowland and Wooding, 1975). C. sputorum ss sputorum can be isolated from the human gingival margins (Loesche, Gibbons and Socransky, 1965). C. sputorum ss bubulus may be isolated from the genital tract of male or female cattle and sheep (Smibert, 1974), the original isolation being from the preputial sac of normal bulls (Florent, 1953).

C. sputorum ss mucosalis is the intracellular vibrio described in association with PIA (Rowland, Lawson and Maxwell, 1973; Lawson and Rowland, 1974). It can be distinguished from C. coli, other members of the genus Campylobacter and of the ss C. sputorum (Lawson, Rowland and Wooding, 1975). It has been isolated from a number of enteropathies (Rowland and Lawson, 1975b) and is implicated in PHE (Rowland and Lawson, 1975a). Although demonstrable in affected tissue in PIA, RI, NE and PHE, it has not been found in the intestines of healthy pigs or pigs with other enteric disorders (Lawson and Rowland, 1974). It has been isolated from the mouths of pigs without gross changes of PIA (Lawson, Rowland and Roberts, 1975). As has already been described the bacteria lie free within the apical cytoplasm of the epithelial cells and do not appear to invoke any inflammatory reaction. A similar relationship has been described in Sweden (Martinsson et al., 1974; Jonsson and Martinsson, 1976) and mucosalis isolated from cases of PIA (Gunnarsson et al., 1976). A preliminary study of the surface antigens of PIA has been reported (Lawson, Rowland and Roberts, 1976).

Two outbreaks of vibrio infection in pigs differing from swine dysentery have been described (Birrel, 1957; Radostits and Finn, 1969). Insufficient information is given to identify the organism in either case but from the diseases described it is possible that C. sputorum ss mucosalis was involved. Birrel (1957) demonstrated the organisms in carbol-gentian-violet or diluted carbol-fuchsin stained smears. He described the vibrios in small groups, usually near tissue cells; these were possibly groups of intracellular mucosalis. A highly fatal necrotic-haemorrhagic-fibrinous ileitis is described by Radostits and Finn (1969). The disease has features resembling PHE (Rowland and Rowntree, 1972).

The classification and differentiation of the genus *Campylobacter* is presented in Table I (after Smibert, 1974). C. sputorum ss mucosalis is proposed as a third subspecies of C. sputorum (Lawson, Rowland and Wooding, 1975). This division of the genus *Campylobacter* ignores C. coli (Vibrio coli) first described by Doyle (1944). C. fetus ss jejuni and C. fecalis conform to the description of C. coli given by others except Doyle, whose isolates differed in their nitrate reduction from those described since the original description. Lawson, Rowland and Wooding (1975) recognised that C. coli strains vary in their characters and proposed division into three types for this heterogeneous group. These workers use the epithet C. coli to include all the porcine catalase positive campylobacters whilst realising that some may be identical with other named species. This nomenclature will be followed by the writer.

Other Chronic Enteropathies and Intestinal Neoplasia in the Pig

Porcine Paratuberculosis

The first suspected case of paratuberculosis in the pig was

Table I The Classification and Differentiation of the Genus *Campylobacter* (After Smibert, 1974).

	catalase	nitrate reduction	nitrite reduction	H ₂ S on T S I	H ₂ S, lead acetate strips	1% glycine	3.5% NaCl	25 C
1a. <i>C. fetus</i> subsp. <i>fetus</i>	+	+	-	-	-	-	-	+
b. <i>C. fetus</i> subsp. <i>intestinalis</i>	+	+	-	-	+	+	-	+
c. <i>C. fetus</i> subsp. <i>jejuni</i>	+	+	-	-	+	+	-	-
2a. <i>C. sputorum</i> subsp. <i>sputorum</i>	-	+	+	+	+	+	-	+
b. <i>C. sputorum</i> subsp. <i>bubulus</i>	-	+	+	+	+	+	+	d
3. <i>C. fecalis</i>	+	+	?	+	+	+	d	-

reported by Runnels (1955). A ten month old animal was involved, and the terminal twelve inches of the ileum and most of the caecum and colon had a thickened, corrugated mucosa. Histologically the lamina propria and sub-mucosa were infiltrated with large numbers of histiocytic cells, some of which were multinucleate and occasionally formed large foreign-body type giant cells. These cells were shown to contain large numbers of acid-fast bacilli. Ringdal (1963) described tuberculous mesenteric lymph nodes and the recovery of Mycobacterium paratuberculosis from his cases, the infection apparently originated among the cattle on the same farm. Experimental infection of pigs with M. paratuberculosis resulted in a granulomatous enteritis in the ileum (Jorgensen, 1969; Larsen, Moon and Merkal, 1971). Occasionally this condition might have a gross resemblance to PIA.

Porcine Rectal Strictures

In this condition there was an annular cicatrization of the rectal wall, cranial to the anorectal junction. The reported clinical features were poor growth rate, emaciation, intermittent diarrhoea and in addition most pigs had severe enteric disease four to eight weeks before the development of strictures (Wilcock and Olander, 1977a). At necropsy the colon was dilated and there was a fibrinous peritonitis. It has been suggested that Salmonella typhimurium, by producing a prolonged necrotizing colitis, caused the ischaemic damage necessary for the development of the rectal stricture (Wilcock and Olander, 1977b). Although the site is different the lesions in this condition have some features in common with those described in RI.

Intestinal Neoplasia

Head (1976) considered that "adenocarcinoma of the intestine of pigs is a rare, and possibly non-existent condition"; and its absence in studies on the incidence of neoplasia in domestic animals supports this view. Recently six cases of intestinal carcinoma were reported in pigs (Vitovec, 1977). These were found at meat inspection in mature and older animals. Five of the cases were in the middle or distal third of the jejunum, and were constrictive annular growths with stenosis of the intestine and superficial ulceration. The tumours extended through the muscle layers into the sub-serosa and adjacent mesentery, where they formed well-defined nodules. In three cases there were metastases to the regional lymph nodes, but in only one to the lungs. The other case was a mucoid adenocarcinoma of the caecum, with infiltrative growth and metastasis to the regional lymph nodes and lungs. The relationship of these, if any, to PIA is unknown.

Comparison with Human and Other Animal Enteropathies

Emsbo (1951) stressed that PIA/RI in the pig was very similar to Crohn's disease in man, a view shared by others (Crohn and Turner, 1952; Rahko and Saloniemi, 1972a), but refuted by many workers (Field, Buntain and Jennings, 1953; Nielsen, 1955; Westendorp, 1965). In man, Crohn's disease may macroscopically present in three patterns; aphthous ulceration of the mucosa may predominate, strictures can develop or there may be a cobblestone appearance to the mucous membrane (Morson and Dawson, 1972). Involvement of any part of the bowel can occur and the designation regional enteritis is often used. Histologically there is a transmural inflammation with widening of the submucosa by

oedema, the inflammatory infiltrate is predominantly lymphocytic with some plasma cells. In 50% to 70% of cases there is a sarcoid or tuberculoid reaction in the affected tissues and regional glands. The granulomas and the fissuring ulceration are described as being of pathognomonic importance (Morson and Dawson, 1972). Although grossly there may be some resemblance between Crohn's disease and PIA/RI the histological picture described for Crohn's disease in man is distinct from that seen in PIA/RI. The epithelial hyperplasia seen in the latter conditions has not been described in Crohn's disease. This may be due to its absence, it may have been missed, or not manifest clinically.

In E.M. studies of Crohn's disease material (Ranlov, Nielsen and Wanstrup, 1972; Cook and Turnbull, 1975) intracellular bacteria were not described as in the pig (Rowland, Lawson and Maxwell, 1973; Martinsson et al., 1974). Aluwihare (1971) described intramural bacteria in 6 out of 11 cases of Crohn's disease examined. These were seen in the lamina propria and submucosa of areas of bowel not obviously diseased, with intact epithelium and minimal inflammatory changes. No organisms were seen in the epithelium and no bacteria, bacterial remains or viruses were seen in phagolysosomes observed in the epithelial cells themselves.

The aetiology of PIA, and of Crohn's disease in man, have remained obscure for many years, although an infectious agent has been sought in both conditions. Recent experimental evidence has implicated an infectious aetiology for Crohn's disease (Cave, Mitchell and Brooke, 1975; Mitchell, Rees and Goswami, 1976). A small RNA virus has been isolated from Crohn's disease tissue (Aronson et al., 1975; Gitnick,

Arthur and Shibata, 1976) and partial characterization suggests that it is a picornavirus (Gitnick and Rosen, 1976).

Recently, further ultrastructural evidence for the presence of virus-like particles has been presented (Riemann, 1977), and reovirus-like agents have been isolated from the intestinal resections of six out of ten patients with Crohn's disease (Whorwell et al., 1977). The virus isolated by Whorwell et al. was an RNA virus, which was antigenically related to the Nebraska calf diarrhoea virus, and may be a member of the rotavirus group. These authors stress that its isolation does not necessarily prove a causal relationship, but suggest that of the various aetiological agents implicated the best candidate would be a widely distributed organism such as the ubiquitous rotavirus, to which some people may exhibit an abnormal response, analogous to the relationship between measles and subacute sclerosing panencephalitis. De Groote et al., (1977) were unable to show substantial differences in rotavirus antibody titres between patients with Crohn's disease or ulcerative colitis and controls, using Nebraska calf rotavirus as antigen. They suggest that a clarification of the role of viruses in Crohn's disease would profit most from the further characterisation of the various isolates reported on by different groups, a search for viral agents in the human gut, and virological studies in animals, in which successful transmission can be obtained. Bacterial variants, L-forms, have also been suggested as an aetiological agent in Crohn's disease (Parent and Mitchell, 1976a, b).

Added evidence for the presence of a cell wall defective pseudomonas-like bacterial variant in Crohn's disease was presented by Parent and Mitchell (1977), who, using a 0.45 μ filter found the organism

regularly in lesions of the disease and isolated it in pure culture on artificial media. Also injection of bacterial L-forms into the wall of the rabbit ileum produced a granulomatous response (Orr et al., 1974). Epidemiological studies do not support the concept of an infectious disease (Miller et al., 1975; 1976) and also experimental production of lesions, using homogenates of affected tissue in laboratory animals, the basis of the infectious hypothesis, has not been achieved by all workers (Heatley et al., 1975).

Whether or not any of these agents are of primary aetiological significance is far from clear, and the possibility exists that they may just be multiplying in the altered environment provided by the pathological changes in the intestine. An alteration in the immunological status of the affected intestine in Crohn's disease has been reported (Baklien and Brandtzaeg, 1975, 1976). Secondary to severe inflammatory reactions, secretory epithelium may show decreased production of secretory component (SC), the selective transport of SC-stabilized secretory IgA and IgM is thus jeopardised and a vicious circle may be set up in the mucosa (Brandtzaeg and Baklien, 1977). Donnelly, Delaney and Healy (1977) add further to the confusion in presenting evidence for a transmissible factor in Crohn's disease. The injection of rabbits' ileum with homogenates of either normal or Crohn's affected human bowel tissue gave Crohn's-like changes after six months in eleven out of twenty seven animals, but after twelve months the rabbit bowel had returned to normal. They interpreted these results as presenting evidence for a transmissible factor present in both normal and Crohn's affected bowel in the aetiology of Crohn's disease. Clearly other interpretations of this data are possible and the results shed some doubt

on previous reports of successful transmission. Much further careful work will be necessary to elucidate the confused situation which has arisen over the aetiology of, and the infectious agents present in Crohn's disease, but no indication has been found of any similarity to PIA.

Whipple's disease, although very different to PIA in its pathology, has in common with it the constant association of bacteria with the intestinal wall. Whipple's disease is a systemic condition with intestinal changes including an accumulation of PAS positive macrophages in the lamina propria and submucosa, predominantly the former, and in the drainage lymph nodes. Response to antibacterial therapy and the demonstration of bacilli-like bodies in the lamina propria suggest a bacterial aetiology (Kent et al., 1963). A variety of bacteria have been isolated from Whipple's disease tissue and it has been suggested that no one specific bacterium is responsible but rather that some defect in the mononuclear phagocytic cells means that bacteria are not dealt with in the usual manner. Recent work using immunofluorescent techniques indicated however that the bacteria in cases of Whipple's disease were very closely related antigenically (Keren, et al., 1976). Two studies implicated a beta-haemolytic enterococcus (Charache et al., 1966) and an alpha-haemolytic streptococcus (Clancy et al., 1975), the bacteria being cell-wall deficient in both cases. In these last two studies the evidence for the isolated organism being the intracellular bacterium in the cases studied seems sound. The bacteria in Whipple's disease have been described in the epithelium as well as in the lamina propria and within macrophages (Dobbins and Ruffin, 1967; Watson and Haubrich, 1969).

In the epithelium the bacteria were in lower, but appreciable numbers than in the lamina propria. They were located mostly towards the base of the epithelium and usually between the cells, in the extracellular spaces. Intracellular bacteria were either encapsulated in a single or double membrane, within phagosomes, or apparently free within the cytoplasm of the epithelial cell (Watson and Haubrich, 1969).

No description of a condition in humans exactly comparable to PIA has been found. Histopathologically it would seem that PIA most closely resembles the benign intestinal adenoma of human pathology. Bacteria have been implicated indirectly in human gastro-intestinal neoplasia, certain clostridia producing a carcinogen or co-carcinogen from bile acids, but no direct association, as in PIA, has been described. Patients with large bowel cancer had both a high faecal bile-acid concentration and the appropriate clostridia in comparison to controls (Hill et al., 1975).

Regional enteritis has been described in the dog (Strande, Sommers and Petrak, 1954; Kruiningen, 1972) and a histiocytic ulcerative colitis of Boxer dogs has been documented (Kruiningen, 1965), the latter resembling Whipple's disease in the human. An infectious aetiology for this granulomatous colitis of Boxer dogs has been suggested (Kruiningen, 1967). The agent involved was a lipid-rich, ribosome-rich, coccoid to coccobacillary organism, which possessed a cell membrane and often a nucleoid element, and ranged from 100 to 500 nm in size (Kruiningen, 1975). These organisms have only been described in the macrophages of the affected colon. A case of transmural ileocolitis induced by *Trichuris* has been described (Widmer and Kruiningen 1974) which resembled regional enteritis.

It would seem that although a number of chronic enteritides of the dog occur, which may have a gross resemblance to PIA or RI, and in which micro-organisms can be demonstrated in the affected tissue on occasions, none of these conditions is analogous to PIA or the other porcine proliferative enteropathies.

In sheep a terminal ileitis has been described (Cross, Smith and Parker, 1973). The condition was characterised by poor weight gains, and a thickened terminal ileum, grossly resembling John's disease. Histologically there was an epithelial hyperplasia which in areas gave the mucosa an adenomatous appearance. Aetiology was undetermined. This condition seems to be very similar in clinical manifestations, gross and histopathology to PIA but unfortunately no E.M. studies were reported on the affected tissues. Intestinal polyps have been described in the jejunum of a 3 month old lamb (Helfer and Koller, 1976). Histologically the polyps were epithelial proliferations of the mucosa, and contained coccidial forms. The authors suggested that they differed from the papilliform growths occasionally described in coccidiosis in sheep and lambs, and that they were an incidental finding and afforded a suitable environment for coccidial multiplication. Again no E.M. studies were reported, so the presence or absence of intracellular bacteria in the epithelium of these polyps is unknown.

Hoorens et al. (1977) described a case of regional enteritis in a four month old lamb, which was in poor condition and had a slow growth rate. At necropsy the distal jejunum and the ileum were thickened and the mucosa had a cobblestone appearance. The histological appearance, as assessed from their illustrations, was similar to that of

porcine RI. By electron microscopy bacteria were demonstrated in the apical cytoplasm of the intestinal epithelial cells. It is interesting that a vibrio has been reported in association with scouring and wasting in hoggets in New Zealand (Russel, 1955). No gross changes were seen, and histopathology was not mentioned. In smears of the intestinal mucosa and of faeces, stained with dilute carbol fuschin, there were many vibrios seen. Vibrios were isolated in an atmosphere containing 10% CO₂, at 37°C, but using cultures the disease could not be reproduced experimentally.

A proliferative ileitis has been described in hamsters (Jonas, Tomita and Wyand, 1965; Boothe and Cheville, 1967) in which there was a markedly hyperplastic, immature epithelium. In the epithelial cells of affected ileum, bacteria have been demonstrated lying free within the apical cytoplasm (Wagner, Owens and Troutt, 1973). These bacteria have been seen dividing in this site and tentatively identified as E. coli 0138 closely resembling Shigella boydii types 11 and 12 (Wagner et al., 1973). Clinically the condition is referred to as "wet-tail" and affected animals exhibit diarrhoea and weight loss. Some animals recover and the condition appears to behave clinically like an infectious disease.

It will be immediately apparent that this condition resembles closely PIA, both in its pathology and in the presence of bacteria free within the cytoplasm of epithelial cells in affected tissue. Moreover its behaviour suggesting an infectious disease resembles that reported for PIA. As with PIA, no degenerative or inflammatory change associated with the presence of the intracellular bacteria is described.

An intraepithelial vibrio associated with acute typhilitis of young rabbits has been described (Moon et al., 1974). In a retrospective study the ultrastructural pathology of a diarrhoeal disease in weanling rabbits was studied. The authors found intracellular vibrios mostly within membrane-bound or partially membrane-bound vacuoles in the cytoplasm, and occasionally free in the cytoplasm. Since this was a retrospective investigation it was not possible to fully elucidate the role played by the vibrio in the diarrhoeal disease. It was suggested however that some vibrio can invade caecal epithelium and cause or contribute to diarrhoeal disease in young rabbits.

A haemorrhagic-necrotic enteritis has been described in a baboon due to C. (Vibrio) fetus (Boncyk, Brack and Kalter, 1972). Human infections with C. fetus have also been described, which are probably of alimentary origin (Butzler, et al., 1976). The association between Campylobacters and human enteritis has recently been recognised. In this, large numbers of Campylobacter fetus subspp. jejuni were present in the faeces of diarrhoeic patients (Skirrow, 1977). It was reported that spread was rapid between young children and to their mothers, but not between adults. Dogs can also become infected and pass on the disease (Skirrow, 1977).

A granulomatous enteritis has been described in the horse (Cimprich, 1974), and Corynebacterium equi enteritis in the foal (Cimprich and Rooney, 1977). In the latter condition, the mucosa of the large intestine was thickened, and in the lamina propria and submucosa there were P.A.S.-positive macrophages containing small, pleomorphic, Gram-positive bacilli. C. equi was cultured from the intestinal mucosa of these cases and demonstrated free and within macrophages in the lamina propria by

by electron microscopy. Muscular hypertrophy of the ileum in a horse has also been described (Rooney and Jeffcott, 1968), and muscular hypertrophy with diverticulosis has been well documented in the pony (Rider, 1930, 1932). Rider described a syndrome of capricious appetite and progressive wasting in pit ponies. At necropsy there was a thickening of the bowel wall involving both the mucosa and muscular coats. The mucosa was thrown into folds described as resembling the lesion in Johne's disease in cattle. Occasionally diverticula were present as well as the marked thickening of the wall. Epithelial hyperplasia was not described. The incidence was reported to be higher in Icelandic ponies but was described in other breeds. Aetiology was unknown. This condition resembles PIA in a number of its features but no description of epithelial hyperplasia is given. A number of possible reasons for this exist; the disease may not have epithelial hyperplasia as a feature at any stage, the cases presented for P.M. examination were mostly of a chronic nature when its recognition may have been difficult, or its presence may have been missed.

Tyzzer's disease due to an obligate intracellular bacterium, Bacillus piliformis, (Ganaway, Allen and Moore, 1971) has been described in a number of species. Lesions were described in the intestine, liver and myocardium; in the intestine they involve the ileum and large intestine. This condition seems to be of intestinal origin and the liver lesions are periportal in origin. A transmissible enterocolitis in hamsters due to B. piliformis has been described (Nakayama et al., 1975) and B. piliformis has been implicated in the aetiology of megaloileitis in rats (Jonas, Percy and Craft, 1970). B. piliformis lies free within the

cytoplasm of the affected cell (Port, Richter and Moize, 1971; Goto et al., 1974) and the lesion is necrotising in character. Nakayama et al., (1976) in typhlohepatitis in hamsters produced by oral inoculation with Tyzzer's organism noted that "in many parts of the intestines, organisms were present in seemingly living epithelial cells, rather than within necrotic foci as observed in the liver." Tyzzer's disease has been described in the horse (Swerczek, 1977) and it appears that foals acquire infection through the ingestion of faeces from adult horses which are excreting B. piliformis (Swerczek, 1977). A case of intestinal Tyzzer's disease and Spirochaetosis in a guinea pig has been described (McLeod et al., 1977) in a thin, diarrhoeic, unthrifty guinea pig. Bacillus piliformis was illustrated free within intestinal epithelial cells, and in the related intestinal crypts large numbers of spirochaetes were present. The unusual possibility was raised by the authors that the spirochaetes invade the intestinal epithelium, lose their outer envelope, extend their axial filaments, and then resemble B. piliformis.

A transmissible colonic mucosal hyperplasia, due to a variant of Citrobacter freundii, has been described (Barthold et al., 1976). Inoculation of the organism into germ-free mice induced colonic mucosal hyperplasia, and it was recovered in pure culture from the affected animals. The colons were usually rigid and thickened, and the number of cells lining the crypts of hyperplastic colons was two to three times greater than in controls. Goblet cells were decreased in number or absent, and the mitotic rate of the epithelium was increased. Hyperplasia, without inflammation, occurred in the earlier lesions and inflammatory cell infiltrates were irregularly present at later stages. The location of C. freundii was not reported.

The Possible Role of Vitamins in Proliferative Enteropathies

The role of vitamins in NE of pigs has been suggested frequently. Nicotinic acid (Davis and Freeman, 1940; Runnels, Monlux and Monlux, 1965; Hungerford, 1975) and pantothenic acid (Goodwin, 1962) have both been implicated. A pathological study of niacin deficiency in swine has been reported (Dunne et al., 1949). Changes involved the large intestine, the wall of which was thickened and the histopathology was described as being characterized by an extensive mucoid degeneration. The crypts were distended with mucus and the goblet cell population of mucosa was showing increased secretory activity. In a study of the pathology of pantothenic acid deficiency in pigs (Sharma et al., 1952) the authors described changes mostly involving the large intestine although the terminal small intestine was reportedly involved at later stages. The wall was thickened and oedematous and histologically there were areas of epithelial necrosis, desquamation and superficial ulceration, and the remaining epithelium was distended with mucus. In neither case does the histology conform to that described by Rowland and Lawson (1975b), in which all their cases of NE demonstrated an underlying hyperplastic epithelium lacking goblet cells. Goodwin (1962) described cases of PIA and RI occurring in herds in which animals were also showing the classical symptoms of goose stepping and hair loss brought about by pantothenic acid deficiency.

An interesting report is that of a pig herd problem of unthriftiness supposedly due to niacin deficiency (Madison, Miller and Keith, 1939). The pigs were anorexic and unthrifty and over 50% of the herd had died. Niacin supplementation was started and the pigs were reported

to recover entirely within six weeks. The authors suggested that this does not occur in swine practice to any great extent, but that occasional pigs were seen with the symptoms. No pathology was reported but the whole history and signs of this outbreak were compatible with it being PIA.

The role of vitamins is difficult to evaluate in published work because no information is available about the presence or absence of PIA in the herds investigated or in the pigs used experimentally. Similarly no information is available about the occurrence of mucosalis infection in the pig population at present, although indications are that it is very widespread. So although these observations are of interest and help to emphasise the confusion surrounding many enteropathies of the pig, they leave unanswered the question of the role of vitamins in this condition.

It has been reported that hamsters are markedly affected by niacin deficiency (Decker and Henderson, 1959) and on an experimental niacin deficient diet the animals lost weight, half of them died and the remainder developed diarrhoea. Hamsters from the same group but receiving niacin supplementation grew normally and remained healthy. These observations have led authors to suggest that niacin deficiency may be of aetiological significance in proliferative ileitis in hamsters.

A focal avillous hyperplasia of the mouse duodenum on a diet deficient in pantothenic acid has been reported (Seronde, 1970). The glandular epithelium was hyperplastic, immature and at the deeper levels anaplastic.

Vitamin deficiency predisposing to intestinal infection or

increasing the severity of intestinal infection is not unknown. Folic acid deficiency in the guinea pig increased the severity of intestinal shigellosis (Haltalin *et al.*, 1970).

Competition between an intracellular parasite and the host cell for a specific amino-acid occurs (Hatch, 1975). This competition for host pools of isoleucine was a limiting factor in chlamydial multiplication.

These suggest possible ways in which vitamin deficiency could operate if it has a role in the pathogenesis or in the predisposition to PIA.

The Possible Role of Mineral Deficiency in PIA

Of the trace elements only zinc has been implicated. Martinsson and Ekman (1974) reported low zinc levels in post-weaning, wasting pigs, which on post-mortem examination had terminal ileitis, and response to zinc therapy was said to be good. Addition of zinc at 150 to 250 grams of zinc sulphate per ton of food, or 5 grams per 20 to 40 litres of drinking water is often used, as supporting therapy, in weaned or fattening pigs with a wasting syndrome in Sweden (Martinsson and Ekman, 1976). It was shown that unless there was a dietary zinc deficiency evident these levels of zinc did not improve daily weight gain (Martinsson and Ekman, 1976). Martinsson, Ekman and Jonsson (1976) also reported low zinc levels in their cases of regional ileitis, but they noted that in most of the herds investigated there was no correlation between a low content of zinc in the feed and the occurrence of wasting pigs or pigs with RI. They therefore suggested that the low zinc levels were secondary manifestations of the disease.

Zinc deficiency in the pig has been described and the characteristic lesion is that of parakeratosis, involving the epidermal layer of the skin (Whitehair and Miller, 1975). The intake of food is also markedly reduced, the rate of weight gain impaired and there may even be a weight loss.

In man zinc deficiency has been reported as a complication of Crohn's disease (McClain, 1977), in which it has been suggested that the anorexia and growth retardation may be secondary to a chronic zinc deficiency. These signs are common in zinc deficiency, and in children low levels of zinc have been correlated with anorexia, poor growth and hypogeusia (Hambidge *et al.*, 1972). Response to zinc therapy is good.

The ultrastructure of the intestinal epithelium in zinc-deficient rats was reported by Koo and Turk (1977), the cells were mature, although showing some degenerative changes.

At present it is unclear if zinc is involved in the pathogenesis of PIA, since deficiency may be a consequence of the enteropathy or a predisposing factor to the establishment of mucosalis in the intestinal mucosa.

Host-parasite interactions with respect to micro-organisms and the intestinal epithelium

In recent years this has become a field of very active investigation. Takeuchi (1971, 1975) divides the enteric micro-organisms into four groups depending on their invasive potential into the host. The first group includes pathogens which readily penetrate the epithelial barrier, multiply in the mucosa and lead to systemic invasion, e.g. Salmonella (Takeuchi and Sprinz, 1967) and Entamoeba histolytica (Takeuchi

and Phillips, 1975). The second group includes Shigella (Takeuchi et al., 1965; Takeuchi, Formal and Sprinz, 1968), which tend to reside and multiply in the epithelial cells, especially of the luminal surface, diminishing in numbers towards the crypts. Few Shigellae are seen in the lamina propria and systemic invasion seldom occurs. The third group resides preferentially in the brush-border of the epithelial cell and do not invade deeper, e.g. cryptosporidia (Vetterling, Takeuchi and Madden, 1971), Gardia (Morecki and Parker, 1967) and spirochaetes (Takeuchi and Zeller, 1972). The fourth group does not attach, produces symptoms through elaboration of an enterotoxin, e.g. certain serotypes of enterotoxigenic E. coli.

The group one type of micro-organisms exemplified by salmonella cause a series of cytoplasmic degenerative changes in the intestinal epithelium and there is also an active response to the penetrating organisms by the gut mucosa. The bacteria are located within vacuoles in the cell cytoplasm. The second group, e.g. Shigella, produce much more severe cytoplasmic damage in the intestinal epithelium. Bacteria are described in single or double membrane-bound vacuoles and also free in the cytoplasm. Lateral spread from intestinal epithelial cell to cell has been described (Ogawa, 1970), and dysentery bacilli can divide in this specific environment. In the third group spirochaetes populate the intestinal epithelium of primates replacing the glycocalyx, destroying most microvilli and causing an attenuation of the terminal web, but the remaining cytoplasmic features are unaltered (Takeuchi and Zeller, 1972). In the fourth group no structural alterations are noted in the epithelium but changes at a biochemical level result in an outpouring

of fluid into the lumen of the alimentary tract. Although these divisions do not necessarily fully accord with those made by others they are a useful starting point.

The host-parasite relationship considered in this work does not fit into Takeuchi's classification. Mucosalis is present in the cytoplasm of the epithelial cell, it can survive and multiply in this site and it lies free within the cytoplasm of the cell, not bound by membranes of the host cell. It causes no degenerative changes in the epithelial cells parasitized, and no inflammatory response in the surrounding mucosa.

By analogy with other enteric micro-organisms it would seem that a number of phases are possibly involved in the establishment of this relationship; attachment, which may be specific, to the enteric epithelium; penetration into the epithelial cell and survival and multiplication within the cytoplasm of the epithelial cell. Although these stages have not been elucidated for C. sputorum ss mucosalis it may be of interest to consider them here. Attachment to intestinal epithelial cells has been described for E. coli with K88 antigen (Jones and Rutter, 1972), Vibrio cholerae (Jones, 1975) and Salmonella (Tannock, Blumershteyn and Savage, 1975; Duguid, Darekar and Wheater, 1977). Mucosalis has been shown to adhere to isolated epithelial cell brush borders and a number of cells types (Lawson - unpublished results). Penetration has been described at an ultrastructural level for E. coli (Staley, Jones and Corley, 1969b) and Salmonella (Takeuchi, 1967; Takeuchi and Sprinz, 1967). In both cases uptake seemed to be by phagocytosis and the organisms were in membrane-bound vacuoles. Mucosalis lies free

within the host cell cytoplasm and so, if entry is by phagocytosis, the bacteria must subsequently gain release from the phagosome. Penetration directly into the cytoplasm of non-phagocytic cells has been described for trypanosoma cruzi (Tanowitz et al., 1975). Studies of in vitro interaction between Rickettsia mooseri and human peripheral macrophages (Andrese and Wisseman, 1971) described the uptake of the rickettsia by phagocytosis and its subsequent escape from the phagosome to replicate free within the cytoplasm of the cell. With Toxoplasma gondii there is controversy as to whether entry of the parasite into cells is by phagocytosis (Jones, Yeh and Hirsch, 1972) or direct penetration (Zaman and Colley, 1972). Lycke, Carlberg and Norrby (1975) consider that an active penetration through the cytoplasmic membrane of the host cell is the mode of entry of Toxoplasma merozoites and they describe a penetration-enhancing factor, produced by the organism, which enzymically modifies the host-cell membrane.

Once in an intracellular position mucosalis is able to survive and multiply but the factors involved in, and important in this are not understood. The lack of degenerative change and inflammatory response is unusual for a bacterial infection. The similarity to the situation in proliferative ileitis in the hamster has already been mentioned. Other bacteria free within the host-cell cytoplasm are known, e.g. Bacillus piliformis, the causal agent of Tyzzer's disease, free within the cytoplasm of hepatocytes in the Mongolian gerbil (Port, Richter and Maize, 1971) but in this case the character of the lesion is necrotizing.

With reference to enteric infections there are a number of ultra-structural studies on the uptake of bacteria and viruses by the intestinal

epithelium. A specialisation of the villous absorptive cell in the duodenum (Staley, et al., 1969a), jejunum (Staley, et al., 1968) and ileum (Staley, 1969; Moon, 1972) in the neonatal pig and in other species has been described. Many of these studies have been interested particularly in colostrum absorption, but work with adenoviruses in mice (Worthington and Graney, 1973a, 1973b) and TGE virus in piglets (Wagner, Beamer and Rustic, 1973) have shown this apical tubular system to be involved in virus uptake. The tubular system is a function of cell age and is present for a longer period in the porcine ileum post-natally than in the upper small intestine (Moon, 1972). Uptake of particulate antigens and bacteria by the epithelium overlying the intestinal lymphoid tissue, Peyer's patches has been described (Bockman and Cooper, 1973). Recently a new cell type, the M-cell, was described ultrastructurally and it was suggested that this cell was possibly involved in uptake of particulate antigen (Owen and Jones, 1974).

Of interest is the fact that in those cases of intestinal penetration by, or uptake of, micro-organisms it is the villous or surface epithelium which is involved. For E. coli, in monocontaminated colostrum-deprived pigs, attachment and uptake took place on the villous epithelium in the ileum, and on the surface epithelium in the colon (Staley et al., 1969d; Staley, Corley and Jones, 1970). Similarly in experimental Shigella infection in rhesus monkeys the surface epithelium was involved initially, and by cell to cell spread the crypt epithelium became involved (Ogawa, 1970). With TGE virus in pigs (Pensaert, Haelterman and Hinsman, 1970), coronaviruses in calves and dogs (Mebus et al., 1973; Takeuchi, et al., 1976) and rotaviruses in calves and pigs (Hall et al., 1976) it was the villous epithelium which was involved, whereas in



in intestinal adenovirus infection in mice the crypt epithelium was involved as well as the villous epithelium (Takeuchi and Hashimoto, 1976).

C. sputorum ss mucosalis is situated in epithelium which ultra-structurally resembles undifferentiated crypt cells, the marked immaturity of this epithelium being a characteristic feature of the condition. Such epithelium would lack the apical tubular system.

This unique relationship may have important implications in a wider biological field, with the interest in the evolutionary acquisition of organelles by eukaryotic cells. It has been suggested that a symbiotic relationship between simple prokaryotic cells and the eukaryotic cells of higher plants and animals may have been the starting point for this. This in effect implies that the organelles such as chloroplasts and mitochondria of higher plants and animals have been acquired from prokaryotic cells of the groups that today contain bacteria and algae. Evidence for this has recently been reviewed (Margulis, 1975, 1976). John and Whatley (1975), in a study of Paracoccus denitrificans put forward a hypothesis for the origin of the inner mitochondrial membrane from a bacterial plasma membrane, based on the functional similarities between this organism and mitochondria.

CHAPTER II

PLAN OF WORK AND GENERAL MATERIALS AND METHODSPlan of Work

In this work field cases of PIA and other proliferative enteropathies were studied, initially to provide experience for the author in working with the techniques involved, and to continue to monitor the naturally occurring disease. In the course of this, PIA was unexpectedly encountered in an M.D. herd, and subsequently this herd was studied in some detail in an attempt to monitor the occurrence of PIA in a small closed herd.

Pigs were exposed experimentally to Campylobacter sputorum subsp. mucosalis, isolated from field cases of PIA, to study the host-bacterial relationship.

Transmission experiments were undertaken, using material from naturally occurring cases of PIA, in initial attempts to reproduce the disease. The design of these experiments took into account observations made in the earlier work.

The recovery phase of PIA was also studied, using both field and experimental cases. PHE cases were investigated in detail by a number of techniques, to try to elucidate the pathogenesis of this condition.

General Materials and Methods
Experimental Animals

The piglets used in these experiments were obtained from three local farms. Post-mortem examinations are routinely carried out on stock from these premises and the health status of the herds are therefore

known. The majority of the sows were obtained from a local breeding research organisation (herd A), in the herd of which PIA has been recognised at a low level over a number of years. This herd has no major disease problems. One sow was obtained from a small conventional herd (X), which was used to provide red blood cells for blood group typing. Whether or not PIA was present in this herd is unknown. Sows were introduced into the isolation unit a few days before farrowing.

Weaned pigs were obtained from the M.D. herd at the University of Edinburgh Veterinary Field Station (herd D), and a College of Agriculture Pig Unit (herd B). Later in this study it is demonstrated that PIA is present in herd D and it was in this herd that the outbreak of PHE and PIA described by Rowland and Rowntree (1972) occurred. Herd B, in which various managerial investigations are undertaken has a number of disease problems, and PIA has been endemic for a number of years. Coli-bacillosis was a major problem in the past, but is seen only infrequently at present.

Further details of the animals and their management will be recorded in the appropriate chapters. All animals were observed at least once daily, but more often on several occasions each day. On each occasion, prior to the introduction of animals to the isolation unit, the pens were cleaned, disinfected with an approved carbolic disinfectant and formalin fumigated (B. Vet. Codex, 1953).

Culture Media

The following media were used:

Blood Agar (B.A.) - Hartley's digest agar (Cruickshank, 1965) containing

5% defibrinated horse blood.

Columbia Blood Agar (C.B.A.) - Columbia Agar Base (Oxoid, CM 331) incorporating 7% defibrinated horse blood.

Reinforced Clostridial Medium (R.C.M.) - Reinforced clostridial medium (Oxoid, CM 149).

Tryptose Phosphate Broth (T.P.B.) - Tryptose Phosphate Broth (Oxoid, CM 283).

Serum Agar - Blood Agar Base No. 2 (Oxoid, CM 271) containing 10% inactivated horse serum.

The Oxoid media were prepared according to the manufacturer's recommendations.

Diphasic slopes (C.B.A./T.P.B.) were prepared by overlaying a 30 ml C.B.A. slope, in a 100 mls medical flat, with approximately 30 mls of T.P.B.

Novobiocin brilliant green blood agar (N.B.G.) - Blood Agar Base No. 2 (Oxoid, CM 271) containing 5% defibrinated horse blood, Novobiocin (Upjohn Ltd., Crawley, Sussex) at 5 ugs per ml and brilliant green (G.T. Gurr, Ltd., London) at a dilution of 1/60,000.

Novobiocin brilliant green blood agar + Naladixic Acid (N.B.G. + N) - As N.B.G. plus naladixic acid (Dev. 719 Winthrop Laboratories) at 20 ugs/ml.

For N.B.G. the blood agar base was dissolved by boiling and then autoclaved at 120 lbs per sq. in. for 15 mins. After cooling to 56°C blood, novobiocin and brilliant green were added.

For N.B.G. + N the naladixic acid was added after boiling to dissolve the agar but before autoclaving for 15 mins. at 120 lbs per sq. in. Blood, novobiocin and brilliant green were added after cooling to 56°C as for N.B.G.

N.B.G. is recommended by Lawson and Rowland (1974) as a selective medium for the isolation of Campylobacter sputorum ss mucosalis and C. coli. It allows the growth of porcine campylobacters while inhibiting the growth of many other bacteria. When N.B.G. plates were used, either C.B.A. or B.A. plates were usually inoculated in parallel.

Initially batches of brilliant green solution were titrated and appropriate dilutions chosen so that counts of mucosalis on N.B.G. were within one \log_{10} of counts on non-inhibitory media. Despite this procedure one batch of N.B.G. plates was prepared which appeared to be considerably more inhibitory than expected. This phenomenon was investigated (Appendix I) and from that time onwards each newly poured batch of N.B.G. plates was checked using two test strains 1248/72 2C2 and 106/75.

Bacterial Strains

In the early experiments a culture of Campylobacter sputorum ss mucosalis was obtained from Dr. G.H.K. Lawson, Department of Veterinary Pathology, University of Edinburgh Veterinary Field Station. This strain had originally been isolated on 10th June 1974, from a field case of intestinal adenomatosis in a 15 week old pig. The reference number of this organism is 604/74/LI-3. Since its isolation from the large intestinal mucosa this organism had been maintained by sub-culture on to B.A. at 10 day intervals. Serologically and biochemically this organism is considered to be typical of the subspecies mucosalis.

In later experiments a different strain (106/75/SI-10⁻³) was used. This organism was isolated on 30th January 1975, from a case of

adenomatosis in a 14 week old female Duroc pig (See Chapter III). The organism used for the experimental work was isolated on non-inhibitory medium and was purified twice from a single colony and then immediately prepared for storage at -80°C in a number of aliquots. On each occasion that pigs were exposed to this organism a fresh aliquot was removed from the -80°C and this was used as the source of the inoculum for the infecting dose. 106/75/SI-10⁻³ is biochemically and serologically typical of the subspecies mucosalis (Lawson, Rowland and Wooding, 1975) and retained these properties on storage and recovery from -80°C .

Storage of Cultures at -80°C

The medium used for this was Hartley's digest medium (Cruickshank, 1965) with serum added. This was used with approximately 17% added tyndalised glycerol.

24 hour cultures on solid media (B.A. or C.B.A.) were used as the source of bacteria to be stored. A loopful of the culture was added to approximately 1 ml of the storage medium in a sterile vacutainer and the top replaced. Within ten minutes of preparation the suspension of organisms in glycerinated broth was placed in the -80°C .

To recover the organism the -80°C stored cultures were thawed at room temperatures and a few drops immediately inoculated onto a B.A. or C.B.A. plate.

Antisera

Hyperimmune antisera prepared against whole cells of C. sputorum ss mucosalis were obtained from Dr. Lawson. These sera ("OH") had been

prepared by the inoculation of rabbits with the surface growth from 48 h blood agar plate cultures suspended in normal saline. Four inoculations were made at 3 - 5 day intervals and the rabbits were bled 7 days after the last injection. The serum used in slide agglutination tests was preserved with 30% glycerine. The antiserum used in slide and tube agglutination tests was prepared against the neotype strain of C. sputorum ss mucosalis 1248/72 (NCTC 11000).

Fluorescein isothiocyanate (F.I.T.C.) conjugated goat anti-rabbit globulin serum was also obtained from Dr. Lawson. This serum had been prepared by the hyperimmunisation of goats with a crude preparation of rabbit gamma-globulin. The goat serum was precipitated with "Rivanol" and saturated ammonium sulphate (Mostratos and Beswick, 1969) and the purified globulin conjugated with F.I.T.C. (Nairn, 1964). The conjugated serum was subsequently absorbed with fresh, washed, porcine red blood cells and acetone dried porcine liver powder.

Sera were stored at -20°C except for those aliquots in current use which were stored at $+4^{\circ}\text{C}$.

Miscellaneous Materials

Millipore Filters (Millipore U.K. Ltd.)

25 mm diameter, 1.2 μm pore size Millipore filters were used to filter the mouth swab samples after they had been shaken in sterile saline.

25 mm diameter, 0.8 μm pore size Millipore filters with 2 pre-filters (Whatman 2.1 cms diameter GF/C Glass Fibre) were used to filter samples of intestinal contents suspended in 0.1 M. pH. 7.2 P.B.S.

Swabs

Cotton wool swabs on wooden sticks, which had been hot-air sterilised, were used to sample the oral cavity.

Streptomycin Solution

The streptomycin solution for oral administration was prepared from 'Dimycin' (Glaxo Laboratories Ltd., Greenford, Middlesex). This was administered at a dose rate of approximately 5 mgs streptomycin and 5 mgs dihydrostreptomycin per Kg body weight.

'Engemycin' (Mycofarm Ltd., Braintree, Essex) containing 50 mgs oxytetracycline (as the hydrochloride B.P.) per ml was administered by intramuscular injection to the sows K and L at the manufacturer's recommended dose level.

'Cyfac 25'* (Agricultural Division, Cyanamid of Great Britain Ltd.) was used for the oral medication of the sows K and L and their litters. Each 1 lb (453 grammes) of Cyfac 25 contains the equivalent of 16.6 grammes of Aureomycin, Chlortetracycline hydrochloride (as animal feed intermediate), 16.6 grammes sulphadimidine and 8.3 grammes of procaine penicillin, in a cereal diluent. Cyfac 25 was administered at the higher dose level recommended by the manufacturers, i.e. equivalent to 6 gms Cyfac 25 per 10 Kgs liveweight daily.

Chalk Suspension

This was prepared immediately before use by suspending 1 gm of chalk powder in 5 mls of distilled water.

Benzetimide⁺ Solution

Benzetimide was administered orally at a dosage of 0.250 mgs/Kg. (Marsboom, Temmerman and Symoens, 1973).

* Kindly donated by Cyanamid.

+ Kindly donated by Jansen Pharmaceuticals.

Diluting Fluids

Sterile saline (S.S.) containing 0.85% sodium chloride.

Sterile phosphate buffered saline (P.B.S.) prepared using stock phosphate buffer solution (Cruickshank, 1965) and 0.85% sodium chloride. This was used at 0.1 M or 0.01 M concentrations, both pH 7.2.

Source of Mucosa Used in Transmission Experiments

The mucosa used in the transmission experiments (Chapters 7 and 8) was obtained, with one exception, from clinical cases of PIA, which are documented in Chapter 3. Details of these pigs and the recovery of mucosalis from them are given in Chapter 3. The pig not described in Chapter 3 from which mucosa was used for oral dosing of the litter of sow F in Chapter 7 is detailed below.

Pig 71/76

This pig was obtained from herd B. It was 9 weeks old and had been on medication with tylosin ('Tylan', Elanco Products Ltd.) until two days previously. It was from a group of weaners, two of which had not been thriving and had been removed from the group and treated with tylosin. Both pigs had lost condition considerably, but the other one had improved and 71/76 had been brighter, more alert and showing an improved appetite for two days.

When sow F farrowed difficulty was experienced in obtaining pigs showing signs typical of PIA, so this animal was considered for this purpose.

At post-mortem examination no abnormality was seen in the small

intestine but it was thought that the mucosa of the large intestine was possibly thickened. Histologically, cryostat sections showed no frank adenomatous change of the large intestine examined. Since it had not proved possible to locate other animals to provide infectious material, despite considerable doubts as to its suitability it was decided to proceed using this material for oral dosing. Subsequently mucosalis was not recovered from the large intestinal mucosa. In direct smears of the large intestinal contents *Balantium coli* was seen, and also spirochaetes, only the minority of which resembled Treponema hyodysenteriae.

Incubation of Media

All plates were incubated at 37°C. Incubation was carried out under microaerophilic conditions unless otherwise stated. Plates and bottles were placed in a Mackintosh and Fildes anaerobic jar, without a catalyst. The jar was evacuated to a negative pressure of 650 mms of mercury, allowed to stand for at least 10 minutes and the negative pressure checked before adding hydrogen; 10% of this atmosphere was removed and replaced by CO₂. Two holes were pierced in the side of the plastic petri dishes to allow improved gaseous exchange, in all plates incubated microaerophilically. Bottled media were stoppered with a cotton wool plug.

Preparation of Suspensions of *C. Sputorum* ss *Mucosalis* for Oral Administration

Two or three days before a mucosalis suspension would probably be required for oral dosing, a fresh aliquot of 106/75 was removed from the -80°C and handled as already described. After twenty-four hours

the purity of growth on B.A. or C.B.A. plates was checked by Gram-stained smears and by slide agglutination using 1248/72 2C2 OH anti-sera. If the culture was morphologically pure and serologically typical, the growth from this plate would then be used to inoculate diphasic C.B.A./T.P. slopes. These were incubated under the microaerophilic conditions and the gaseous environment changed every 24 hours. 24 h, 48 h or 72 h old cultures were checked by Gram-stained smear for morphology and purity, phase contrast for motility and a slide agglutination test, to confirm that the cultures were mucosalis. Appropriate cultures were pooled to prepare the inoculating dose.

From the bulked suspension 1 ml was removed and added to 9 mls sterile 0.1 M.P.B.S. Log_{10} serial dilutions were made in P.B.S. and 0.02 ml drops plated on B.A., incubated microaerophilically at 37°C and the colony forming units counted (Miles and Misra, 1938).

The inoculum was used to dose the pigs as soon as possible after its preparation.

Preparation of Mucosa from Field Cases of PIA for Use in Transmission Studies

Immediately after euthanasia of the pig the abdominal viscera were removed for examination. From grossly thickened areas of terminal ileum or large intestine material was taken for histological, fluorescence and E.M. examination. To confirm that the lesion was that of PIA, H. & E. stained cryostat sections were examined and also impression smears of the mucosa stained by the Brucella differential technique.

The remainder of the affected intestine was used to provide material for oral dosing in the transmission studies:-

The gut was opened in a sterile tray using sterile instruments and the surface of the mucosa washed using sterile 0.01 M P.B.S. The mucosa was then scraped from the tissue using a sterile scalpel held at 90° to the surface of the tissue. The mucosa was weighed and mixed with an equal volume of sterile tryptose phosphate broth and homogenised using an M.S.E. homogeniser. 1 gm of mucosa was also homogenised with 19mls of sterile R.C.M and used in the manner described later to estimate the numbers of mucosalis present in the mucosa.

The homogenised mucosa was used to dose the pigs as soon as possible after its preparation.

With the bulked mucosa from pigs 87/76 and 88/76 half of the prepared mucosal suspension in tryptose phosphate broth was stored at -80°C immediately after its preparation.

When this stored mucosal suspension was required for use it was removed from the -80°C and placed in an incubator at 37°C. Immediately it had thawed 1 ml of the suspension was removed and added to 19 mls of sterile R.C.M. and 1/20 dilutions made in R.C.M. This was used to estimate the numbers of mucosalis present. The remaining thawed mucosal suspension was used immediately for oral dosing of piglets.

Oral Dosing of Piglets

Chalk

This was poured into the back of the piglet's oral cavity, with the head held back. The piglet's nose was held until the chalk suspension had been swallowed.

Benzetimide

The Benzetimide solution was administered using a sterile syringe and short intra-gastric tube.

C. sputorum ss mucosalis

Cultures of C. sputorum ss mucosalis were administered orally to the pigs using a sterile syringe and a short intra-gastric tube.

Mucosa

Mucosal suspensions were administered to the piglets either using a sterile syringe and a short intra-gastric tube or by pouring the suspension to the back of the pig's mouth with the head held back. The mouth was then held closed until the suspension had been swallowed.

Collection and Bacteriological Examination of Gingival Swabs

The gingival margin was thoroughly massaged with a cotton wool swab. The swab was then placed into 5 mls of sterile saline and shaken for at least 5 minutes. This fluid was filtered using a 1.2 μ Millipore filter. The last few drops were inoculated onto inhibitory and non-inhibitory, i.e. N.B.G. and B.A. respectively, and streaked out conventionally. Incubation, selection of colonies and identification of mucosalis was carried out in a similar manner to that employed with intestinal mucosa.

Euthanasia

Young piglets were killed by the inoculation of "Expiral" (Pentobarbitone Sodium B. Vet. C., 200 mgs/ml.- Abbott Laboratories Ltd., Queenborough, Kent) into the anterior vena cava or heart and then

bled out immediately by severing the carotid arteries. Older pigs were stunned electrically and then similarly bled out.

Necropsy

Immediately after euthanasia and collection of blood samples the carcass was placed in dorsal recumbency. A mid-line incision was made from the xiphoid cartilage to the cranial border of the pubis and the skin reflected laterally. The abdominal cavity was opened by incising the linea alba; the alimentary tract from the oesophagus, at its point of emergence through the diaphragm, to the rectum was removed along with the liver and spleen, after sectioning at these extremes. After cutting the mesentery the alimentary tract was opened out and along with the associated lymph nodes examined. At predetermined sites samples were taken for bacteriological, histological, immunofluorescent and E.M. examination. The same procedure was carried out at each site. First a loop of intestine of approximately 15 to 20 cms length was ligated at each end and removed after sectioning adjacent to the ligatures. The immediately distal 1 cm to 1.5 cms portion of intestine was then removed and opened. It was laid flat on a clear glass slide and samples for electron microscopy and immunofluorescence taken. The 15 to 20 cms of intestine distal to this was washed out, filled and placed in 10% buffered formol-saline. After the samples had been taken the remaining alimentary tract was opened and the mucosa examined. The carcass was examined for other pathological abnormalities and to determine the animal's general condition. Any tissues showing gross changes were sampled as appropriate for bacteriology or fixed in 10% buffered formol-saline for histology.

Piglets dying during the course of the experimental work were all subjected to post-mortem examination and sampled as appropriate, taking into consideration the lesions, and the time since death of the animal.

The sites from which samples were taken are shown in Figure 1. In early experiments both contents and mucosa were sampled at all 5 sites. In the later work the mucosa at sites, 1, 3 and 4 were examined, these sites are referred to as M.S.I. T.S.I. and L.I.

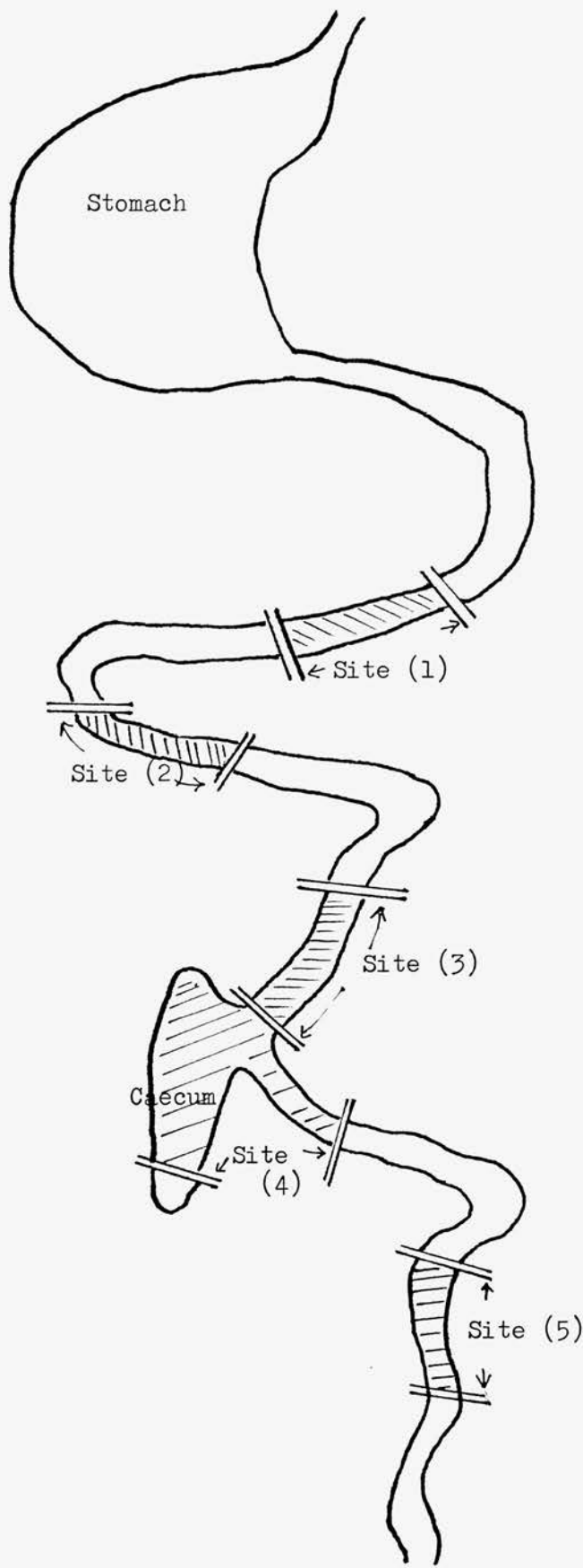
- Site (1) Mid-way between the pylorus and the ileo-caeco-colic valve. Corresponds to the mid-small intestine, i.e. M.S.I.
- Site (2) Mid-way between sites (1) and (3).
- Site (3) The terminal ileum, corresponds to the terminal small intestine, i.e. T.S.I.
- Site (4) Situated between the middle of the body of the caecum extending to approximately 10 cms along the proximal colon. This corresponds to the large intestine, i.e. L.I.
- Site (5) Situated approximately one-third of the way along the proximal colon.

Bacteriological Techniques

The bacteriological samples were collected in clean sterile trays. Manipulation of the samples was carried out in these using sterile instruments.

In those cases in which intestinal contents were examined, the

Figure 1 Sites from which samples were taken.



loop of the intestine was sectioned at one end and the open end flamed. One gram of the contents, or an otherwise known amount, was added to 9 mls of sterile 0.1 M P.B.S. Ten-fold dilutions were then made in sterile 0.1 M P.B.S.

The loop of intestine was next opened and the surface of the mucosa washed using 0.01M P.B.S. Once the mucosal surface was free of visible debris the mucosa was scraped off using a scalpel held at 90° to the surface. One gram of mucosa, or another measured weight, was added to approximately 10 mls sterile R.C.M. The mucosa in R.C.M. was then homogenised in a blender (M.S.E. 77313) at maximum r.p.m. for 30 secs. The volume of R.C.M. was made up to 19 mls and twenty-fold dilutions prepared in sterile R.C.M.

The isolation and estimation of viable mucosalis was made by inoculating 0.1 ml amounts of appropriate dilutions on to C.B.A. or B.A. and N.B.G. plates and spreading the inoculum with a sterile glass spreader.

In a number of cases filtration of mucosal samples was carried out. One gram of mucosa was removed as described above and homogenised in 9 mls of 0.1M P.B.S. The resultant suspension was filtered using a 0.8 μ Millipore filter with two prefilters. As much of the mucosal suspension as possible was filtered and 0.1 ml amounts of the filtrate were spread out on plates as described above.

Plates were incubated microaerophilically at 37°C for 48 h, with the atmosphere being changed at 24 h. At 48 h, a count of the number of colonies was made and dependent on colonial type, representative colonies were sub-cultured on to B.A. All plates were incubated for at

least 5 days before discarding except in the case of plates showing confluent growth of organisms or the growth of moulds. The colonies were recounted after reincubation.

From consideration of the total counts and those that proved to be mucosalis, estimates were made of the numbers of mucosalis present in the mucosa.

Identification of *Campylobacter Sputorum* ss *Mucosalis*

Sub-cultures from the primary plates on to B.A. were examined by Gram-stained smear after 24 h or 48 h incubation. Those which could be classified as vibrios on a morphological basis were further examined by a slide catalase and a slide agglutination test. Colonial pigment was also noted. Those isolates which were morphologically vibrios, catalase negative, and reacted in slide agglutination tests with 1248/72 2C2 OH antiserum were considered to be mucosalis. A selected number of isolates were examined in slope catalase and tube agglutination tests, after purification twice from single colonies.

Slide Catalase Test

Bacterial growth was carefully removed from the plate and gently emulsified in a drop of 3% hydrogen peroxide solution on a clean glass slide. If effervescence was not seen within two minutes a negative result was recorded.

Slope Catalase Test

This test was carried out on 48 h cultures on serum agar slopes (Cowan and Steel, 1965).

Slide Agglutination Tests

Bacterial growth was emulsified in 0.85% sterile saline on a clean glass slide. A drop of antiserum was added to the emulsion with a platinum loop. The slide was gently tilted from side to side and examined against a dark background using a hand lens.

Tube Agglutination Tests

The organism under test was grown on a C.B.A. slope for 48 h, checked for purity by Gram-stained smear, and the growth washed off with 0.3% formol-saline. The antigen suspension was centrifuged and washed three times and resuspended in a small amount of 0.3% formol-saline. The suspension was diluted with 0.85% sterile saline to a density equivalent to Brown's Opacity Tube No. 2, estimated by nephelometer. Doubling dilutions of hyperimmune antiserum were prepared and unit volumes of antigen were added to unit volumes of the serum dilutions. A unit volume of antigen was added to a unit volume of 0.85% saline as a negative control. Tubes were placed in a 56°C water-bath for 30 secs. to allow mixing and incubated at 37°C for 18 h. A control antigen was tested at the same time, in an identical manner. The result was recorded as the amount of deposit visible to the naked eye, the end point being the highest dilution of serum to give 50% agglutination.

Light Microscopy

Fixation and Processing

All tissues were fixed in 10% formol-saline, processed routinely, embedded in paraffin wax and sections cut at 5 μ .

Histological Stains

Sections from all blocks were routinely stained with alum haematoxylin (Cole, 1943) and eosin (H. & E.) for initial histological examination. For demonstration of intracellular bacteria special stains were employed: Young's modification of Warthin-Starry Method for spirochaetes (Young, 1969) and Levaditi's method for spirochaetes (Levaditi and Manouelian, 1906). Gomori's methenamine silver method was also used (Gomori, 1946). Other special stains employed included: Martius Scarlet Blue, Masson's trichrome, Gordon's and Sweet's for reticulin, carbol chromotrope, Phloxine tartrazine, and the feulgen reaction for deoxyribonucleic acid (D.N.A.) (Drury and Wallington, 1967).

Impression Smears of Mucosa stained modified Ziehl-Neelsen

Impression smears were made on clean, dry, microscope slides and fixed by air-drying and stained as follows:

Dilute carbol fuchsin	-	5 minutes
Acetic Acid 0.5%	-	25 seconds
Methylene Blue	-	10 - 15 seconds

Fluorescence Microscopy

Blocks of tissue 5 x 5 x 5 mm were snap-frozen and stored in liquid nitrogen. Sections were cut at 8 μ on a cryostat microtome (Slee, London) operating at -25°C. A section was stained with H. & E. to confirm its suitability and then parallel sections were stained for immunofluorescence microscopy.

Known positive material from confirmed PIA cases and negative material were always stained in parallel with sections under study.

Staining

Air-dried sections were overlaid with mucosalis antiserum and incubated in a moist chamber at 37°C for 30 mins. They were then washed in 0.01M P.B.S., pH 7.2, for 15 mins with two changes of P.B.S. The sections were then stained with F.I.T.C. conjugated goat anti-rabbit globulin serum for a further 30 mins at 37°C in the moist chamber. Finally, they were washed twice in 0.01M P.B.S. pH 7.2, at 5 min intervals and then rinsed in distilled water and dried.

Sections were examined on a "Wild M20" microscope (Wild, Heerbrugg, Switzerland) using transmitted blue light from an H.B. 200 mercury vapour lamp with a 2 mm B.G. 38 and a 2 mm B.G. 12 filter. Occasionally, if greater resolution was required sections were examined on a "Leitz Orthoplan" (Leitz, Wetzlar, Germany) using incident blue light from an H.B. 200 mercury vapour lamp with a 4 mm B.G. 38 filter.

Electron Microscopy

Fixation, Processing and Staining

Blocks, approximately 2 x 1 x 1 mm, were selected for processing for electron microscopic examination. Fixation was carried out in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.3, for 24 h at 4°C. The blocks were then rinsed in buffer and post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.3 for 1 to 2 h. After rinsing in buffer the blocks were then dehydrated through graded ethanols and embedded in Araldite (Polaron Ltd.).

Thin sections were cut at 0.5 μ on a Cambridge-Huxley Ultratome using glass knives and stained with 2% thiamine/0.5% toluidine blue in 1% borax at 60°C and examined by light microscopy to select areas for

electron microscopic study. Ultrathin sections were cut at 600 - 1200 Å, mounted on copper grids and stained as follows:-

lead citrate (Reynolds, 1963)	- 2 minutes
0.02 N sodium hydroxide	- 20 seconds
distilled water	- 10 seconds
uranyl acetate (sat. solution in 50% ethanol)	- 10 minutes
50% ethanol	- 20 seconds

Grids were examined at 50KV on an A.E.I. - EM6B electron microscope and electron micrographs were taken on Ilford EM 4 photograph plates; or on a Phillips EM100 electron microscope at 60 KV and electron micrographs taken using 35 mm Ilfadata B. type A504 film (Ilford Limited, Ilford, Essex).

CHAPTER III

STUDIES OF THE NATURALLY OCCURRING DISEASEPART A. FIELD CASES OF PROLIFERATIVE ENTEROPATHIESINTRODUCTION

Although considerable progress has been made in recent years in the understanding of porcine enteric disease, there has remained a group of conditions of ill-defined aetiology over which there has been much confusion. These enteric disorders have been considered in Chapter I. They usually involve the terminal small and large intestine, which are thickened and may additionally be necrotic or haemorrhagic. In PIA/RI many workers have described the inflammatory nature of the lesion (Emsbo, 1951; Field, Buntain and Jennings, 1953; Rahko and Saloniemi, 1972a; and others), or the muscular hypertrophy (Nielsen, 1955) and it is only in recent years that the underlying epithelial proliferation in the mucosa has been stressed (Dodd, 1968; Rowland and Rowntree, 1972; Rowland and Lawson, 1974), although the adenomatous nature of the change was recognised in early work (Biester and Schwarte, 1931; Biester, Schwarte and Eveleth, 1939). Other changes have been reported inconstantly. Changes in the ganglia of the gut wall have been described (Westendorp, 1965), and the presence of giant cells and the formation of epithelioid granulomas have been documented (Emsbo, 1951; Rahko and Saloniemi, 1972a).

PIA and RI are well described in the literature, and also necrotic enteritis has been recognised for many years in all areas of the world

where pigs are kept (see Chapter I). It was not until 1975 however, that a relationship between PIA, RI and NE was suggested (Rowland and Lawson, 1975).

Despite the recognition of these conditions for many years their aetiology has remained unresolved. Recently intracellular bacteria have been described in constant association with cases of PIA in the United Kingdom (Rowland, Lawson and Maxwell, 1973; Rowland and Lawson, 1974; Lawson and Rowland, 1974) and Sweden (Martinsson *et al.* 1974; Jonsson and Martinsson, 1976). The bacteria have been isolated, characterised and named Campylobacter sputorum subsp mucosalis (Lawson, Rowland and Wooding, 1975). Mucosalis has also been recovered from cases of RI and NE (Rowland and Lawson, 1975b). Viruses have not been described in the reports of the ultrastructure of PIA (Rowland and Lawson, 1974; Jonsson and Martinsson, 1976), but in a report on the isolation of reo-like viruses from pigs' faeces Bergeland *et al.* (1975) described the recovery of these viruses from some, but not all cases of PIA. However, they were also present in faeces from a wide-range of pigs with enteric and other diseases. Their significance is therefore unknown, and to date there has been no evidence put forward demonstrating the presence of viruses in the lesions of PIA.

A study of naturally occurring cases of proliferative enteropathies in the pig was undertaken for a number of reasons. To allow the author to become familiar with the changes of PIA, RI and NE and to obtain bacteriological expertise in handling material from these conditions. This allowed the writer to assess the relationship between these conditions described by Rowland and Lawson (1975b), and also provided

material for use in transmission studies and strains of mucosalis for infectivity experiments. No virological techniques were employed in this study, but in examining tissues from cases ultrastructurally a search was made for virus particles.

MATERIALS AND METHODS

Animals

The pigs examined were field cases obtained from local farms for which the Pathology Department of the University of Edinburgh Veterinary Field Station provides a diagnostic pathology service. Some of the cases were presented for diagnosis, others were, on request, provided live from farms on which PIA is known to occur. For comparison to provide control material intestines were collected from pigs submitted suffering from diseases of other organs, most commonly respiratory disease.

Necropsy examination

In cases presented dead a routine necropsy examination was carried out, as described in Chapter II. Cases presented live were killed, bled out and immediately necropsied.

Histological examination

Tissue was fixed, processed, and sections stained as described in Chapter II. Histological examination was carried out on grossly affected areas of intestine, and also areas which did not appear involved to the naked eye. Equivalent samples were taken from control pigs.

Immunofluorescence and Electron Microscopy

Samples of tissue from adjacent areas to those taken for histological examination, were fixed or frozen, and processed as described in Chapter II. Equivalent material was obtained from control pigs.

Bacteriological examination

This was carried out as soon as possible after euthanasia, and quantitation of mucosalis undertaken using the methods detailed in Chapter II. In animals dead for some time before necropsy, the mucosa of the intestine was washed, sampled and inoculated onto non-inhibitory (B.A.) and inhibitory (B.B.G.) media. Quantitation was not normally attempted on this type of material. Mucosalis was identified and the numbers estimated as described in Chapter II.

Impression smears of the washed mucosa were made and stained by the modified Ziehl-Neelsen method.

RESULTS

Clinical Signs/History

The pigs were mostly from farms in which PIA was known to occur and the animals were culled for failure to thrive. These pigs were post-weaned, and in the six to twenty weeks age range. Occasional pigs may have shown some sign of diarrhoea, but this was not a usual presenting sign for uncomplicated adenomatosis. Cases of NE were often "found dead", after a period of failing to thrive and especially terminally these cases may have shown some evidence of scouring.

Those animals used as controls were most commonly suffering from respiratory disease.

Pathological Findings

Gross Findings

Control Pigs

In control pigs there was no evidence of thickening of the intestinal wall at any level and the mucosa was not thrown into exaggerated folding. Peyer's patches were clearly visible, especially in the terminal ileum (Figure 1).

Pigs with proliferative enteropathies

All pigs presented were in poor bodily condition, which was in some cases severe enough to be termed emaciation. Pathological changes were detected only in the intestinal tract, the severity and degree of change was variable. The classification and terminology of Rowland and Lawson (1975b) was adopted and these cases will therefore be described under three separate headings; PIA, RI and NL.

Although Peyer's patches were clearly visible in the control pigs, they were obviously different from the raised plaques of adenomatous mucosa seen in pigs affected with PIA. On stretching the wall of the intestine the Peyer's patches were flattened out, whereas the exaggerated folding of the mucosa seen in cases of PIA was not effaced by this procedure. On closer examination of Peyer's patches lymphoid follicles were visible to the naked eye.

PIA

In these cases various lengths of the terminal ileum, caecum and colon were involved. In severe cases the abnormality could be recognised from the serosal surface of the gut, whilst in

the least affected histological examination was required to confirm whether or not mucosal abnormalities were present. The affected portions of intestine were thickened and firm, and often there was sub-serosal oedema giving a reticulated appearance to the serosal surface (Figure 2). On opening into the affected portion of intestine the mucosal surface was observed to be thrown into exaggerated folds (Figures 3, 4 and 5), resembling the lesions of Johne's disease in ruminants. These folds often stretched transversely across the wall. Usually the whole mucosa was diffusely involved, but, especially at the periphery of the affected area discrete plaque-like elevations of the mucosa were seen. The plaques were sharply defined and raised above the surrounding normal mucosa. Occasionally polypoid outgrowths of mucosa were seen, especially in the large intestine. It could be appreciated that these polyps were derived from the mucosa and they often had a narrow base. In some cases ridges of the mucosa had undergone necrosis, were grey yellow in colour and still adhered to the underlying surviving mucosa. The lumen content was usually scanty and fluid, with material occasionally adherent to the altered mucosa. The regional lymph nodes were unremarkable, showing only a moderate degree of hyperplasia.

In one case only, pig 355/76, a peritonitis, manifest as a number of soft red nodules two to five millimetres in diameter, was present restricted to the serosal surface of the terminal ileum (Figure 6). These nodules were soft to cut and brown-red in colour. The remaining visceral serosa had a frosted glass rather than the normal translucent appearance, and the adjoining mucosa appeared thickened. No helminths were seen.

RI

The classical descriptive term "hose-pipe gut" was very appropriate for the cases in this group. The terminal ileum was firm, thickened and rigid (Figure 7). The serosa lacked its normal clear lustre and had a more opaque appearance, due to the presence of slight serosal fibrosis on its surface. The mesentery of the affected portion of intestine was often oedematous. On section the external muscle coats were markedly hypertrophied, and the sub-mucosa was thickened due to the presence of granulation tissue, whilst the mucosa was only irregularly present (Figure 8). The inner surface varied appreciably, large areas being lined with granulation tissue in which there were occasional isolated nodules of mucosa (Figure 9). Ulcers were also seen on otherwise healthy areas of the mucosal surface (Figure 9). The lumen of the affected portion of ileum was greatly reduced. The drainage lymph nodes showed marked hyperplasia and were often oedematous.

NE

In this group, as with PIA, the affected portions of intestine varied from pig to pig, but involved the terminal ileum and large intestine either singly or together. The area involved was firm and thickened, and it was possible to recognise the necrosis of the mucosa from the serosal surface (Figure 10). Occasionally the necrosis extended to include Peyer's patches, so that grey-white pin point foci were visible from the serosa (Figure 10). The whole of the affected mucosa was grey-yellow, necrotic and firmly adherent to the underlying surviving mucosa or sub-mucosa (Figure 11). The

sub-mucosa and muscle coats were oedematous. The necrosis of the mucosa involved only the thickened areas of intestine, and where there were thickened plaques present at the periphery of areas of involvement, these were often necrotic too. The drainage lymph nodes were enlarged, hyperplastic, often oedematous and showing evidence of lymphadenitis.

Histopathology

Controls

The small intestines of control pigs had a normal villous architecture (Figure 12). The villous length varied, being longer in the mid small intestine compared to the more distal portions, and was also shorter over the lymphoid nodules of Peyer's patches (Figure 13). The lamina propria was unremarkable, although the number of cells present varied considerably. Lymphocytes, plasma cells, macrophages and often large numbers of eosinophils were present. The cells on the villi were mostly columnar absorptive cells with well developed brush borders, occasional goblet cells were seen. In the crypts immature pyramidal cells predominated, with a few mucus secreting cells also present and very occasional enterochromaffin cells. Paneth cells were not seen.

In the large intestine the normal histological structure was present with elongated crypts opening onto a flattened epithelial surface (Figure 14). The numbers of cells in the lamina propria varied as in the small intestine, but eosinophils were less common.

The epithelial cell types were as in the small intestine, and again Paneth cells were not seen.

PIA

The thickening of the mucosa was seen to be due to an epithelial proliferation. In some areas there were islands of altered glands, sharply demarcated from the surrounding normal mucosa. Villi were usually lost from these areas, which presented a flattened surface. Often the whole mucosa was involved, with loss of normal villous architecture (Figure 15). Areas of adenomatous mucosa overlying Peyer's patches frequently showed aggregations of proliferating immature glands lying within the lymphoid nodules (Figure 16). The appearance, with the enlarged, branched, adenomatous glands opening centrally, was that these nodules had developed from the area of glandular epithelium associated with that particular lymphoid nodule.

In affected areas the epithelium was hyperplastic, the cells being large, immature, non-mucus secreting and often overlapping (Figure 17). The cytoplasm of these cells was eosinophilic, and the nuclei varied from large and vesicular to elongate and densely staining with little internal structure discernible. Occasionally both cell types, i.e., those with open vesicular nuclei and those with spindle-shaped nuclei, were present in the same hyperplastic gland (Figure 18). Mitotic figures were sometimes numerous and occurred throughout the mucosa. The crowded glands were enlarged, elongated and frequently branched. In the large intestine it was often the tips of folds of the mucosa which were involved and in these there was a tendency to form an almost polypoid structure.

Occasional glands contained inflammatory debris and neutrophil polymorphs. The epithelium lining such glands was often flattened, being cuboidal or even more attenuated. In some cases glands had been massively enlarged and contained retained secretions and debris, presumably due to the blockage of the gland's opening on to the surface. The tips of folds of affected mucosa sometimes showed tissue destruction and necrosis. In these areas in the large intestine Balantidium coli and invading bacteria were commonly seen. Associated with this damage there was an infiltration of inflammatory cells. In cases with more extensive surface damage there was often extension of the inflammatory response into the lamina propria and sub-mucosa with an accumulation of cells of the lymphoid series, together with macrophages and neutrophil and eosinophil polymorphs. Active granulation tissue proliferation was a feature of this latter type of case.

Silver-stained sections of affected areas demonstrated the presence of bacteria located within the apical cytoplasm of the epithelial cells. At low power examination this was seen as black staining of the apical cytoplasm (Figure 19), but at higher powers the bacteria could be visualised and their vibrio morphology made out (Figure 20). These bacteria were not present in adjacent normal areas of tissue.

In a number of these cases of PIA a marked dysplasia¹ of the

¹ Dysplasia is used here in preference to anaplasia, since it is considered that the change in PIA is essentially a failure of the epithelial cells to mature. Its transient nature, with complete resolution of the lesion, indicate that it is not truly a neoplastic condition and so it is considered inappropriate to refer to the changes described here as anaplasia.

epithelium, especially of the deeper glands, with infiltration was observed. The infiltrative growth was seen most often, if not exclusively, in those cases in which there was damage to the surface epithelium, and an associated inflammatory response. However, infiltrative growth was not seen in all cases of PIA with surface mucosal damage. Marked variations in the degree of infiltration was seen. In some cases the smooth outline of the glands was lost due to the irregular protrusion of cells into the surrounding lamina propria (Figure 21), but the gland structure was retained. This change occurred in epithelium with a dysplastic appearance, and although this dysplasia was more common at the base of glands it was not confined to this area. A greater degree of infiltration was seen as cords or sheets of epithelial cells extending into the sub-mucosa or underlying lymphoid tissue (Figure 22). These epithelial cells varied in morphology from columnar to cuboidal, or more spindle shaped. Sometimes glands were formed by the infiltrating elements, but often the cells lining these glands were flattened or cuboidal. As few as three cells lined some of these glands, which often had little or no lumen. The infiltrative growth could develop deep into the underlying tissue (Figure 23).

The reaction to these infiltrating tongues of epithelial cells was variable. In some instances there was a marked connective tissue response to the infiltrating cells (Figures 22, 23 and 28), whereas in other cases there seemed to be no connective tissue proliferation and it was not always easy to distinguish between the infiltrating epithelial cells and the surrounding tissue (Figure 24).

Around many of the infiltrating epithelial cells there was no well defined basement membrane.

In silver-stained sections, bacteria of vibrio morphology were identified in the apical cytoplasm of the epithelial cells of the overlying adenomatous mucosa, of those cases showing infiltration. They were also demonstrable in those cases in which the epithelium was dysplastic. Bacteria could also be shown, but in low numbers only, in the infiltrating epithelial cells.

This infiltrative growth often brought the epithelial cells into close proximity to the lymphatic vessels. Infiltration into lymphatics was seen as tongues of epithelial cells protruding into the vessel (Figure 25), or as clumps of cells within lymphatics (Figure 26).

Epithelial infiltration and down growth could easily be distinguished from the close lympho-epithelial relationship between the normal intestinal epithelium and lymphoid tissue of Peyer's patches, seen in the control pigs and in the healthy areas of intestine of pigs affected with PIA. In the normal structure there was downgrowth of the epithelium in the region of, and often quite deep into the lymphoid nodules, so that the epithelium was in intimate contact with the lymphoid cells of these nodules (Figure 13). The epithelium often had a stratified or pseudo-stratified appearance due to the presence of intra-epithelial lymphocytes (Figure 27). The relationship of the adenomatous epithelium with the Peyer's patches varied. Adenomatous glands were seen in association with, and lying deep in the lymphoid tissue. Occasional nodules which resembled

discrete "adenomas" were seen within lymphoid nodules of Peyer's patches (Figure 16). Clumps of dysplastic epithelial cells without gland formation were also seen in lymphoid nodules (Figure 28). Clusters of deeply eosinophilic stained cells with pyknotic nuclei were sometimes associated with areas of infiltrative growth. These aggregations resembled giant cells to some extent, but their appearance more closely related to clumps of degenerating, infiltrating epithelial cells. These cell groups were seen within the lymphoid tissue of the intestine and analogies with foreign-body giant cells (Figure 29) and Langhan's type giant cells (Figure 30) could be drawn.

Occasional isolated glands were seen in the sub-mucosa both in normal intestine and that from cases of PIA, which were not associated with intestinal lymphoid tissue, and in which the cells were well differentiated and the basement membrane clearly visible (Figure 31). This did not appear to be an artefact related to the plane of section and it is unclear whether discontinuity of the muscularis mucosae sometimes allows glands to lie in the sub-mucosa. The sub-mucosa around these glands showed no unusual features.

In uncomplicated adenomatosis the lamina propria contained lymphocytes, plasma cells and a variable number of eosinophil polymorphs, in which features it did not differ from unaffected areas in the same pig or from pigs not suffering from enteric disorders. As already mentioned in cases with tissue destruction and an associated inflammatory response neutrophil polymorphs were present in the lamina propria, the epithelium and the gland lumen, and possibly also in the sub-mucosa. Active granulation tissue proliferation in the lamina

propria and sub-mucosa was sometimes a feature of such cases. The sub-mucosa and muscle coats were normal in most instances.

No abnormalities could be detected in the ganglia of Meissner and Auerbach, in uncomplicated cases of PIA. In those cases with inflammatory changes extending to the deeper layers of the wall, degenerative changes were visible in the neurones of the plexuses, with loss of granularity and increased eosinophilic staining.

The drainage lymph nodes showed a degree of lymphoid hyperplasia, oedema and the presence of eosinophils within the sinuses. No metastases were seen in the drainage lymph nodes of these cases.

In pig 355/76, in which nodules were described on the serosal surface, there were a number of unusual histological features. The nodules on the serosal surface consisted of granulation tissue, with few inflammatory cells present. In the mucosa there were occasional adenomatous glands with a considerable infiltration of eosinophils into the lamina propria of the terminal small intestine, but not in the mid small intestine. There was some oedema of the external muscular layers.

RI

Histologically the lamina propria and sub-mucosa showed a marked granulation tissue proliferation with the luminal surface being covered by necrotic debris containing large numbers of bacteria (Figure 32). Islands of glandular mucosa were only irregularly present and the epithelium in these cases was mostly immature and hyperplastic, although occasional glands were seen with a substantial goblet cell population (Figure 33). Sometimes the surface was covered

by a single layer of cells only, which varied from very flattened to, in some cases, columnar epithelium with occasional goblet cells. The granulation tissue proliferation extended into the muscle layers, which were substantially hypertrophied (Figure 32). As in PIA, silver staining techniques demonstrated the presence of intracellular bacteria aggregated in the apical cytoplasm of the immature, hyperplastic epithelial cells.

The local drainage lymph nodes showed marked lymphoid hyperplasia, oedema and often also a lymphadenitis with the accumulation of neutrophil polymorphs.

Cases intermediate between PIA and RI were seen. These cases showed an adenomatous proliferation of the mucosa with a degree of muscle hypertrophy, infiltration of the lamina propria and sub-mucosa with polymorphs and lymphoid cells and active granulation tissue proliferation.

NE

The lesion in NE was a massive coagulative necrosis of the mucosa, with an associated inflammatory response in the underlying tissue (Figure 34). This inflammatory response varied from acute exudation to a less acute active granulation tissue proliferation. In the underlying or surrounding intact mucosa the outline of enlarged, hyperplastic glands could be seen, although these were often also necrotic. Silver stained sections showed the presence of intracellular bacteria in the underlying intact mucosa, and also demonstrated invading bacteria in the necrotic tissue. Although the bacteria in the apical cytoplasm of intact epithelial cells were vibrios of

uniform morphology, those present in the necrotic tissue varied greatly in form.

There was lymphoid hyperplasia, oedema and lymphadenitis in the local drainage lymph nodes.

Immunofluorescence Results

Controls

In cryostat sections from control pigs, no specific fluorescence was seen in tissues from sites chosen to correspond to those taken from affected pigs.

PIA/RI/NE

In all cases of PIA, RI and NE from which tissue was examined, by staining using hyperimmune mucosalis anti-serum prepared in rabbits, positive bright green particulate fluorescence was seen in the apical cytoplasm of hyperplastic epithelial cells (Figure 35). In sections containing normal glands as well as adenomatous glands, only the latter showed positive fluorescence. The fluorescence was easily recognised due to its position, its brightness and its particulate nature. Specific fluorescence was also occasionally seen in the lamina propria adjacent to adenomatous glands. In that case the fluorescence appeared to be arranged around the periphery of the cytoplasm of cells, probably macrophages (This is considered in detail in Chapter IX).

In both control and affected tissues green autofluorescence of blood vessel walls was seen, and cells with fluorescing orange-yellow granules, which were eosinophils.

Electron Microscopy

Controls

Tissues from control pigs which were examined conformed to previous descriptions of intestinal epithelial cells, already described in Chapter I. No intracellular bacteria were seen, and rarely were bacteria seen in the lumina of glands. These features are illustrated in Figures 36 to 40.

PIA/RI/NE

In toluidine blue stained 1 μ sections it was possible in retrospect to see bacterial bodies in the apical cytoplasm of affected epithelial cells (Figure 41), in some of the material. A low power electron micrograph of an adenomatous gland is illustrated in Figure 42. The adenomatous epithelial cells were ultrastructurally immature, resembling undifferentiated crypt cells (Figures 42 to 44). The cells were elongate and rectangular or pyramidal in shape, with a basal nucleus. The tight junctions and desmosomes between adjacent cells were well developed. The cytoplasm was granular with large numbers of ribosomes, but the membrane systems of the endoplasmic reticulum were less elaborate than in a mature absorptive villus cell. There were accumulations of round, electron dense bodies in the apical cytoplasm of many cells resembling secretory granules (Trier and Rubin, 1965; Toner, Carr and Wyburn, 1971). The mitochondria and nucleus were normal. The terminal web was less well developed than in the mature absorptive cell, and the microvilli at the surface were small, sparse and irregularly distributed. The development of the microvillous border varied with the maturity of the cell.

Bacteria were seen in all the adenomatous tissues examined and were present in many of the affected epithelial cells. They were seen as irregularly curved bodies in the apical cytoplasm (Figures 42 to 44) and although often surrounded by a clear halo, they did not appear to be bound by membranes. The numbers of bacteria varied from only one or two up to between ten and twenty per cell. Depending on the plane of section they showed some variation in size, but were approximately $2.0\text{ }\mu\text{m}$ longitudinally by $0.3\text{ }\mu\text{m}$ in cross-section. Longitudinally the bacteria showed a double curve with rounded extremities, and on cut-surface they were found. Occasional filaments resembling flagella were seen attached to one pole. The bacteria were surrounded by a cell wall, which had a corrugated appearance, of varying thickness or separation from the area of cytoplasmic membrane. The internal structure of the organisms was of an osmiophilic cytoplasm surrounding an electron transparent nuclear zone. Bacteria were occasionally seen dividing in this site (Figure 45).

A search for virus particles was made during electron microscopic examination of these cases. No collections of virions as described in TGE in the pig (Pensaert, Haelterman and Hinsman, 1970; Wagner, Beamer and Ristic, 1973) or in rotavirus infection in the pig (Hall et al., 1976; Lecce, King and Mock, 1976; McNulty et al., 1976) were seen. Small numbers of virus-like particles which were considered to be coated vesicles (Figure 46) (Friend and Farquahar, 1967; Rodewald, 1973) were seen in many sections of the intestine examined both from cases of proliferative enteropathies and from normal pigs.

In one case in which the epithelium showed marked dysplasia and infiltrative growth, electron microscopic examination of areas of Peyer's patches and sub-mucosa was carried out. Deep within a lymphoid follicle epithelial cells were recognised by the attenuated microvilli on their free border, the normal intercellular junctions with desmosomes, and by gland formation (Figure 47). In some cells intracellular bacteria were seen (Figure 48), but the numbers of bacteria were low compared to those in the overlying epithelial cells. The cytoplasmic organelles were less well developed, but were consistent with these cells being undifferentiated epithelial cells. No basement membrane was visible in contact with the epithelial cells, and inflammatory cells were present within the lumen of the gland.

Bacteriological Results

Controls

In modified Ziehl-Neelsen (Z.N.) stained impression smears of the washed mucosa of pigs without alimentary abnormalities no acid fast vibrios were seen. Mucosalis was not recovered from any of these pigs.

PIA/RI/NE cases

In modified Z.N. stained impression smears, made from the surface or cut-surface of the washed mucosa of affected tissue, clumps of acid-fast intracellular vibrios were seen and also occasional free acid-fast vibrios (Figure 49). The acid-fast vibrios were slender, curved rods arranged as clumps usually within the cell, but around groups of cells in the smear free clumps of acid-fast vibrios were seen.

After forty eight hours incubation of the plates inoculated with mucosal samples, colonies resembling those of C. coli or C. sputorum subsp. mucosalis could usually be distinguished. It was found that the colonial growth depended on the hydration of the medium, so that growth either spread across the surface of the medium, formed spurs along the lines of inoculation or formed circumscribed colonies. At forty eight hours incubation, in the absence of excess moisture, colonies of mucosalis did not exceed 1.5 mms in diameter and they were circular, shiny grey and raised with a flat surface (Figure 50). C. coli tended to be more convex and grey-white in colour (Figure 50). If differentiation was not clear after forty eight hours incubation, on further incubation differences in colonial morphology were seen. On prolonged incubation the centre of mucosalis colonies often became depressed, and with time pigment production became an additional criterion allowing differentiation. For C. coli the pigment produced was pink-tan, compared to mucosalis which produced a dirty yellow-green pigment. Recognition of the two was more difficult on N.B.G. plates than on B.A. or C.B.A. plates.

With experience it became possible to tentatively distinguish between mucosalis and C. coli in Gram-stained smears made from the sub-cultures of representative colonies on the primary plates. Mucosalis was usually less heavily stained, more delicate, longer and thinner (Figure 51). C. coli was usually shorter, plumper and more heavily stained (Figure 52). Further distinction was always made, however, on the catalase and serological reactions.

In accordance with Lawson, Rowland and Wooding (1975) it was

always found possible to differentiate mucosalis and C. coli on those characters described below. Catalase positive campylobacters, which produced a pink-tan pigment and did not agglutinate in the slide or tube agglutination tests using 1248/72 2C2 mucosalis anti-serum, were C. coli. Catalase negative campylobacters producing a dirty yellow-green pigment, and which were agglutinated by 1248/72 2C2 mucosalis anti-serum were Campylobacter sputorum subsp. mucosalis. During the course of this study catalase negative campylobacters with characteristics of mucosalis were isolated in large numbers from pigs in two herds. These isolates did not react in slide or tube agglutination tests with the standard mucosalis anti-serum (1248/72 2C2), and these animals are considered separately in Part B of this chapter.

Mucosalis was also recovered from the mouth of a pig with adenomatosis.

The recovery of mucosalis from those pigs used to provide material for transmission experiments (Chapters VII and VIII), or from which mucosalis isolates were obtained for use in infectivity experiments (Chapters IV, V and VI) is summarised in Table 3a.

Selected isolates were subjected to a number of the other biochemical tests described in Chapter II. Mucosalis isolates were found to conform to the original description (Lawson, Rowland and Wooding, 1975). They were catalase negative, H_2S was produced (in T.S.I. and using H_2S paper strips), nitrate and nitrite were both reduced, acid was not produced in carbohydrate media, and they failed to grow aerobically.

In one pig (106/75) in addition to the standard cultural method, the recovery of mucosalis was quantitated after filtration of the

Table 3a Recovery of Mucosalis

Pig Reference	Pathological Classification	Age	Site of Isolation	Numbers of viable <u>Mucosalis</u> per Gram Wet Weight of Tissue
106/75	PIA	14 weeks	Oral cavity	+
			Small Intestinal (mid) mucosa	6.20×10^4
			Small Intestinal (terminal) mucosa	1.34×10^8
			Small Intestinal (terminal) contents	4.54×10^4
			Rectal contents	3.52×10^5
76/76	PIA	12 weeks	T.S.I. mucosa	5.76×10^7
87/76	PIA	12 weeks	T.S.I. mucosa	1.28×10^8
88/76	PIA	12 weeks	T.S.I. mucosa	1.28×10^8
400/76	PIA/RI	14 weeks	T.S.I. mucosa	1.12×10^6

samples. Filtration of the mucosal samples, and also the samples of small intestinal and rectal contents was found to be a useful technique, the numbers of contaminating organisms being considerably reduced. The numbers of mucosalis recovered after filtration were equal to or occasionally greater than the number isolated from unfiltered samples. The success of the technique was shown by the growth of mucosalis from rectal contents; after filtration, on the N.B.G. plate at the $(\frac{1}{10})^2$ dilution there was an almost pure growth of mucosalis, in comparison with the growth from the unfiltered sample, in which mucosalis could not be recognised due to overgrowth by other organisms.

DISCUSSION

This study of field cases of PIA, RI, and NE confirms and extends the observations made previously in this laboratory of the pathology of these conditions, and the constant association of mucosalis with these enteropathies (Rowland and Lawson, 1974; Lawson and Rowland, 1974; Lawson and Rowland, 1975b). A similar relationship has since been reported from Sweden (Martinsson et al., 1974; Jonsson and Martinsson, 1976).

The gross pathology of these cases of proliferative enteropathies was in accord with the descriptions in the literature. The most striking histopathological feature in PIA was the epithelial hyperplasia and marked cellularity of the enlarged glands, the cellular population of which was immature. Two cell types were recognised, one of these was broader with an open vesicular nucleus, the other was narrower and had an elongate, densely staining nucleus. The latter

cell type was the least common, but occasional glands had a low number of these cells present, while other glands had this cell type making a greater contribution, up to half of the total cell population of the gland, and more rarely glands consisting entirely of these cells were seen. The presence of these cells may be an index of the rate of cell division within that epithelium. Mitosis with division of the intestinal epithelial cells takes place adjacent to the gland lumen and after division the daughter cells have to reach out to attach to the basement membrane of the gland (Sandborn, 1970). These cells with elongate, densely staining nuclei were occasionally seen in low numbers in the crypts of normal intestinal mucosa.

Some features recorded by other authors were not seen in this study. Changes in the nerve plexuses of the gut wall were not seen, although they have been described by others (Hoorens, 1962; Westendorp, 1965). In this series of cases any damage to such nerve plexuses was considered as secondary to an inflammatory response in the surrounding tissue.

Giant cells have been described by Emsbo (1951) and Rahko and Saloniemi (1972a). Epithelial infiltrative growth deep into the lymphoid nodules of Peyer's patches occasionally gave rise to collections of epithelial cells with the appearance of giant cells. This was especially so, when these groups of cells had undergone necrosis and were consequently more eosinophilic staining. Giant cells or epithelioid cells were not seen in the affected intestine or drainage lymph nodes in the cases examined in this study. It is possible that the giant cells described by other workers in the gut wall were also groups of infiltrating epithelial cells.

Lymph node metastases were reported by Emsbo (1951) and Nielsen (1971), but were not seen in this series. Infiltrative growth was however seen, especially in those cases with a degree of surface damage, and associated inflammatory response. The infiltrative growth was deep into the underlying lamina propria, submucosa and lymphoid tissue of Peyer's patches. Such cells were often no longer arranged as adenomatous glands, were usually cuboidal rather than columnar, and sometimes even more flattened. Growth into lymphatics was also seen, which is consistent with the observation by others that metastases to drainage lymph nodes occur.

The close relationship between the intestinal epithelial cells and the population of subjacent mesenchymal cells has been described (Pascal, Kaye and Lane, 1968; Kaye, Lane and Pascal, 1968). Possibly some interference with this intimate association takes place, following surface damage or inflammatory changes, which initiates the infiltrative growth seen here. There is a synchrony of replication, migration and differentiation of the epithelial cells and of the population of subjacent mesenchymal cells in the jejunum (Parker, Barnes and Kaye, 1974) and in the colon (Pascal, Kaye and Lane, 1968; Kaye, Lane and Pascal, 1968). Kaye, Pascal and Lane (1971) have reported that the immaturity of the pericryptal fibroblast sheath in adenomatous epithelium, demonstrated by continued fibroblast division at all levels, failure of the morphological maturation of the fibroblasts, and failure of the fibroblasts to secrete their normal extracellular products, collagen and mucopolysaccharides, parallels the degree of immaturity of the overlying adenomatous epithelium.

The rate of cell division in the intestinal epithelium is reputed to be regulated by a feed-back mechanism, in which inhibitory substances, chalones, are produced by the mature villous cells (Rijke et al., 1976). These chalones inhibit cell division in the crypt epithelium. Damage to the surface epithelium may interrupt production of chalones and hence free the crypt cell population from its inhibition, allowing an increase in mitotic activity. Such rapidly proliferating cells may show a greater tendency to dysplasia and infiltrative growth. Such a regulatory mechanism could also play a part in the pathogenesis of the development of the adenomatous lesion. Cells containing mucosalis show a marked immaturity, therefore the population of mature villous cells will be depleted giving rise to the flattened avillous mucosa seen. Subsequently chalones will not be produced and so the epithelium will be released from the negative feedback and there will be an increased rate of cell division. These two factors, removal of the inhibition to replicate and the failure of the parasitised epithelial cells to mature would result in the adenomatous mucosa seen. Whether the cell parasitism by mucosalis initiates the phase of immature growth remains to be ascertained.

The infiltrative growth was distinct from the close lympho-epithelial relationship between the normal intestinal epithelium and the lymphoid tissue of Peyer's patches. This relationship often brought the epithelium deep into the lymphoid follicle, and due to the large numbers of intra-epithelial lymphocytes in this site, the epithelium occasionally had a pseudo-stratified appearance. It was

always possible, however, to distinguish this from infiltrative growth. Submucosal glands in association with the intestinal lymphoid tissue in the pig colon have been reported previously (Biswal, Morill and Dorstewitz, 1954).

Epithelial misplacement (Greene, 1974) and pseudo-carcinomatous invasion (Muto, Bussey and Morson, 1973) have been documented in adenomatous polyps of the colon and rectum, in man. It has been suggested that this is the result of repeated twisting of the stalk of a polyp which causes haemorrhage and this facilitates the passage of non-malignant adenomatous epithelium through the *muscularis mucosae* (Muto, Bussey and Morson, 1973). Of importance in differentiating this from true invasive growth is the presence of lamina propria around the misplaced glands, in contrast to the desmoplastic reaction often seen around invasive epithelium (Helwig, 1959; Muto, Bussey and Morson, 1973; Greene, 1974).

Herniation of the mucosal epithelium into the sub-mucosa in chronic ulcerative colitis in man (Dyson, 1975), and in non-human primates suffering from inflammatory disease of the colon, as well as in apparently normal animals (Scott and Keymer, 1976) has been reported. These are seen in the areas of gut associated lymphoid tissue, where the *muscularis mucosa* is discontinuous. The illustrations resemble the normal lympho-epithelial relationship seen in the porcine gut, which is stressed in this study. Whether there is a species difference with respect to this is not clear. Dyson (1975) states that this mucosal herniation is not involved in the development of dysplasia or cancer. Interestingly epithelial dysplasia and carcinoma occur in chronic ulcerative colitis in man (Cook and Goligher,

1975). In this condition, as in PIA, where cases show infiltrative growth there is mucosal damage and inflammation, and possibly similar factors are involved in the development of these changes in both conditions.

In the series described and illustrated by Rahko and Saloniemi (1972a) the inflammatory nature of the lesion was stressed rather than the adenomatous change. Possibly many of their cases were of the RI type, and in this condition adenomatous glands with immature epithelial cells may be more difficult to find. Often large areas of the mucosa may have a villous pattern and contain a substantial goblet cell population; a thorough search may therefore be necessary to find affected glands.

Ultrastructurally the epithelial cells in affected tissue had features of immaturity and they contained bacterial bodies free within the apical cytoplasm. The relationship between the epithelial cell and the bacteria was as described previously (Rowland, Lawson and Maxwell, 1973; Rowland and Lawson, 1974), the bacteria lying free within the apical cytoplasm and not surrounded by host cell membranes. This host cell-bacterial relationship has also been described in a histologically similar condition in the golden hamster (Wagner, Owens and Troutt, 1973), and in regional enteritis in the lamb bacteria have been reported in the apical cytoplasm of intestinal epithelial cells (Hoorens et al., 1977). Bacteria free within the cytoplasm of hepatocytes in Tyzzer's disease in the Mongolian gerbil have also been reported (Port, Richter and Moize, 1971) but in this condition the lesion is ultimately necrotizing in character.

It is interesting to note that in cases of PIA the adenomatous change involves not only the epithelium overlying the gut associated lymphoid tissue, but also the epithelium outwith these lymphoid areas. The epithelium covering the lymphoid tissue has been described as specialised for the uptake of particulate material, including bacteria (Bockman and Cooper, 1973; Owen and Jones, 1974; Owen, 1977) and in some enteric infections uptake/entry of bacteria is through this specialised epithelium. Such uptake has been described for salmonellosis (Sprinz et al., 1956; Carter and Collins, 1974) and Yersinia enterocolitica (Carter, 1975). Whether this fact means that entry into the epithelium is active on the part of the bacteria, rather than passive is unknown.

Mucosalis was isolated from these cases of all three proliferative enteropathies, as described by Rowland and Lawson (1975b), and the isolates conformed to the previous descriptions (Lawson, Rowland and Wooding, 1975; Lawson, Rowland and Roberts, 1976), with the exception of the serologically distinct variants which will be considered in Part B of this chapter.

No virus particles were seen in material examined ultra-structurally. Virus-like particles, considered to be coated vesicles, were seen but never in collections in vesicles as described in TGE virus infection (Wagner, Beamer and Ristic, 1973) or in rotavirus infection (Hall et al., 1976; Lecce, King and Mock, 1976; McNulty et al., 1976) in the pig. No attempts at viral isolation from any of these cases were made.

The major objectives of this study were achieved in that the writer became familiar with the proliferative enteropathies in the

pig, and with the bacteriological techniques involved in handling the associated bacteria. Isolates of mucosalis and "infective material" were also obtained for the experimental studies which were undertaken. The previous observations made on these conditions were extended, and as a corollary to the study it was possible to assess the value of various methods used in the work, and their possible usefulness in routine diagnostic work.

A number of techniques were found to be of value in the demonstration of mucosalis in affected tissues. Modified A.N. stained impression smears of affected mucosa were extremely useful in providing a rapid indication of the possible presence of mucosalis. This was especially true when the mucosa was only marginally thickened grossly. Immunofluorescence examination of cryostat sections was also a useful technique, but required adequate positive and negative controls. Electron microscopy was suitable to confirm the presence of mucosalis, free within the apical cytoplasm of the intestinal epithelial cells. These last two techniques are very helpful in a detailed study of these conditions, but are probably less useful for routine diagnostic confirmation. Histological examination was used to confirm gross-findings, or in those cases with a suggestive history and no gross lesions, to provide a more definitive diagnosis. Silver-staining of histological sections was unreliable, and if only small numbers of organisms were present more difficult to interpret. Well stained sections did however demonstrate very convincingly the presence of large numbers of intracellular vibrios in the apical region of the affected intestinal epithelium. The Levaditi technique was in most cases superior to the other methods employed.

Cultural examination of fresh material was successful in most cases. Ideally the material to be cultured should be removed from a freshly killed animal. Recoveries were made from material up to seventy two hours after the death of the animal, but the chances of recovering mucosalis probably decrease with the time elapsing after death before sampling. With experience it was possible to identify isolated campylobacters on the basis of the colonies, morphology in Gram-stained smear, slide catalase and slide agglutination tests using 1248/72 2C2 mucosalis anti-sera. As already mentioned the exceptions to this were the cases from which a serologically distinct variant of mucosalis was recovered, and these are described in Part B of this chapter.

Although all these techniques are useful in a study of these enteropathies it is suggested that for routine diagnosis, if herd history, clinical signs, and gross findings are suggestive of PIA (RI or NE), then bacteriological examination of fresh material, histological conformation of the intestinal pathology and examination of modified Z.N. stained impression smears of the mucosa, should be undertaken.

PART B. PORCINE INTESTINAL ADENOMATOSIS ASSOCIATED WITH
SEROLOGICALLY DISTINCT CAMPYLOBACTER SPUTORUM
SUBSPP. MUCOSALIS

INTRODUCTION

A constant association between PIA and an intracellular bacterium has been described (Rowland, Lawson and Maxwell, 1973; Rowland and Lawson, 1974). The bacterium is a vibrio, which has been characterised, and the accepted name is Campylobacter sputorum subspp. mucosalis (Lawson, Rowland and Wooding, 1975). This relationship and the recovery of mucosalis has been detailed in Part A of this chapter.

The organisms are free within the apical cytoplasm of affected intestinal epithelial cells (Rowland and Lawson, 1974), and a similar relationship has been described in affected pigs in Sweden (Martinsson et al., 1974; Jonsson and Martinsson, 1976). Mucosalis isolated by the latter workers (Gunnarsson et al., 1976) has been examined in this laboratory and is serologically closely related to those strains already described (Lawson and Rowland 1974; Lawson, Rowland and Roberts, 1976). Lawson and Rowland (1974) found that an anti-serum prepared against one of the first strains of mucosalis isolated from a case of PIA reacted with all subsequent mucosalis strains examined. Mucosalis isolates recovered from other porcine proliferative enteropathies, RI and NE, were also closely related serologically to the mucosalis strains from PIA cases (Lawson, Rowland and Roberts, 1976).

In this study of field cases of porcine proliferative enteropathies the majority of the isolates of mucosalis conformed to the above description (Part A, this chapter). However, from a number of cases serologically distinct catalase negative campylobacters were recovered and these strains, which differ from those already described, are the subject of this section.

MATERIALS AND METHODS

The pigs in this study were obtained from two herds (B and K). One of these (herd K) had recently been established from the pure Large White M.D. herd (D), which is the subject of the study reported in Part C, of this chapter. The pig from herd B was submitted for diagnostic necropsy, because of wasting to the point of emaciation. Two of the pigs from herd K were received dead for post-mortem examination, the third was submitted live on request.

Bacteriological, immunofluorescent, histological and electron microscopic examination were carried out as detailed in Chapter II.

An anti-serum was prepared in a rabbit against whole organisms (OH) of one isolate from pig 982/76 by Dr. G.H.K. Lawson (Department of Pathology, Edinburgh University Veterinary Field Station) (Lawson and Rowland, 1974). This anti-serum was used in slide and tube agglutination tests, and to stain cryostat sections for immunofluorescence examination.

RESULTS

Clinical Signs/History

The eleven week old pig from herd B was in extremely poor condition, and had wasted to the point of emaciation. This herd has a history of wasting pigs due to PIA, from which mucosalis has been isolated consistently. Mucosalis recovered from this herd had up to that time behaved typically (Lawson, Rowland and Wooding, 1975; Lawson, Rowland and Roberts, 1976).

The other three pigs were from herd K, and two of these were twelve weeks old and were presented for necropsy as sudden deaths in already poor pigs. The third pig was also twelve weeks old, and was presented live on request. It was in poor bodily condition but was not apparently diarrhoeic.

Pathology

The pig from herd B had lesions of PIA involving the large bowel. The two dead pigs from herd K had lesions of NE, whilst the third animal had lesions in the large intestine of PIA. Histological examination confirmed the gross changes, and silver stained sections showed bacteria of vibrio morphology in the apical cytoplasm of cells in affected glands.

Bacteriological Results

Impression smears of the mucosa stained by the modified Z.N. method showed the presence of acid-fast intracellular vibrios within the intestinal epithelial cells. Occasionally, clumps of free acid-fast vibrios were also seen. The pig (982/76) from herd B yielded 4.96×10^6 colonies per gram of adenomatous tissue, which

had the morphological, cultural and biochemical characteristics of mucosalis. Uncharacteristically, however, sub-cultures from these colonies did not react with the standard anti-sera (1248/72 2C2) in slide agglutination tests. In tube agglutination tests "OH" antigens prepared from representative colonies failed to react with 1248/72 2C2 "O" or "OH" anti-sera at dilutions of $\frac{1}{20}$ (homologous titres of the anti-sera 1248/72 $\gg \frac{1}{1280}$). An anti-serum prepared against one strain from this pig (982/76) reacted with the homologous antigen in both slide and tube agglutination (OH) tests, but not with the other strains which react with 1248/72 2C2 anti-sera.

The isolates from the pigs from herd K were similar to those from pig 982/76 and conformed to the morphological and biochemical description of mucosalis. These isolates reacted with the anti-sera prepared against the mucosalis strain from pig 982/76, but not with the anti-sera against other mucosalis strains.

Immunofluorescence Results

Tissue from pig 982/76 had not been taken for immunofluorescent examination. Cryostat sections of the large intestine from pig 1405/76 (herd K) were stained using the anti-serum prepared against the mucosalis strain from pig 982/76. Positive particulate fluorescence was detected in the apical cytoplasm of cells in affected glands but not in the cells of histologically normal glands. Parallel sections stained using the standard mucosalis anti-serum prepared against 1248/72 2C2 were negative. Similarly, immunofluorescent staining of tissues, from which serologically typical mucosalis had been recovered, with 982/76 anti-serum did not give positive fluorescence, whilst parallel sections stained with 1248/72 2C2 anti-serum did show positive fluorescence.

Electron Microscopy

Electron microscopic examination of tissue from pig 1405/76 confirmed the presence of intracellular bacteria in the cytoplasm of the intestinal epithelial cells in affected tissue. These bacteria were free within the cytoplasm and did not appear to be surrounded by host cell membranes, therefore conforming to the host cell - bacterial relationship already described for mucosalis (Rowland and Lawson, 1974). The affected epithelial cells ultrastructurally resembled immature crypt cells (Toner, Carr and Wyburn, 1971).

DISCUSSION

The bacteria isolated from these cases appear to conform to the descriptions of mucosalis but are serologically distinct from those strains of mucosalis already examined (Lawson, Rowland and Wooding, 1975; Lawson, Rowland and Roberts, 1976; Lawson, Rowland and Roberts, 1977b) and considered in Part A, of this chapter. Isolates of mucosalis so far examined from a number of herds, the same herd over a number of years, different regions in the United Kingdom, and from the U.K. and Sweden are serologically closely related, although detectable differences are present (Lawson, Rowland and Roberts, 1977b). The mucosalis strains described here are serologically distinct from those other mucosalis strains examined and there does not appear to be any cross reaction between the two groups with respect to the superficial antigens. The relationship between these serologically distinct groups of mucosalis is as yet

unclear. Variation in the superficial antigens in the mucosalis strains isolated from pigs in one herd over a number of years has been described (Lawson, Rowland and Roberts, 1977b), but these strains are still closely related to each other. Similar antigenic variation has been reported for Campylobacter fetus (Schurig et al., 1973; Corbeil et al., 1975). With experimental infection of virgin heifers with C. fetus subsp. intestinalis, an antigenic drift was described and some of the isolates recovered during the experiment did not react at all with an anti-serum prepared against the infecting strain. The possibility exists that antigenic variation in this group of organisms may be minor with the strains remaining closely related or major, when serological cross reaction is no longer detectable.

Antigenic variation, although better documented in viruses and protozoa has been described in several bacteria; for Streptococcus mutans in the alimentary tract of gnotobiotic rats (Bratthall and Gibbons, 1975) and for vibrio cholerae in gnotobiotic rats (Sack and Miller, 1969; Miller et al., 1972). Fluctuations in the serotypes of the E. coli population of the human intestine has been reported and related to host antibody (Robinet, 1962). For vibrio cholerae there has been a variation in the serotype isolated from field cases with time (Gangarosa, et al., 1967) and in vitro change from one serotype of vibrio cholerae to another has been achieved by incubation in the presence of antibodies (Bhaskaran, 1974).

A further pig killed at the same time as 982/76 and from the same farm, showed changes of PIA and cultural examination

yielded mucosalis serologically typical of the type strain, but distinct from mucosalis strain 982/76. Furthermore, isolates of the two serologically variants of mucosalis have been recovered from the same intestinal mucosal sample in one pig. This was in a piglet infected neonatally with serologically typical mucosalis (Chapter IV).

At present it is suggested that the name mucosalis be retained for those organisms which may be isolated from adenomatous tissue, or serologically and biochemically identical organisms isolated from other sites. To encompass the antigens of the sub-species now requires two anti-sera prepared against the surface antigens of strains 1248/72 2C2 and 982/76, rather than a single anti-serum as previously.

PART C. OBSERVATIONS ON PIA IN A CLOSED MINIMAL DISEASE HERD

INTRODUCTION

There has been no detailed investigation of the incidence of PIA in pig herds. The incidence of PHE has been documented during outbreaks in minimal disease (M.D.) herds (Rowland and Rowntree, 1972; Love, Love and Edwards, 1977), and the contributions to deaths in pig herds made by NE has been reported (Veterinary Investigation Service, 1959 and 1960; Jones, 1969; Pay, 1970a and 1970b). A study of the incidence of PIA is obviously not easy due to the difficulty of diagnosis in the live animal, the presence of asymptomatic cases (Emsbo, 1951; Nielsen, 1971), and the recovery of affected animals (Rowland and Rowntree, 1972).

The herd in which this investigation was carried out (herd D) was the herd of pure Large White pigs established from hysterectomy derived stock, in which a haemorrhagic bowel syndrome associated with PIA was described by Rowland and Rowntree (1972). The latter outbreak occurred in 1965 and lasted for four months. Since that time the herd has been monitored clinically and all deaths subjected to necropsy without evidence of either enteropathy prior to December 1975. In addition an hereditary form of lymphosarcoma has been described in this herd (Head et al., 1974) and a large number of animals necropsied in connection with an investigation of this condition. Towards the end of 1975 the pigs involved in breeding the lymphosarcoma animals, i.e., the carriers, together with their offspring were moved to a separate piggery. This was completed

in early 1976. The building into which these animals were transferred had not been used to house animals for the preceeding eighteen months. In this newly established herd (K) cases of PIA and NE have occurred, in non-lymphosarcoma pigs. These cases yielded the serologically distinct variant of mucosalis, which was the subject of Part B of this chapter.

A number of pigs from the herd (D) were examined in 1975 for the presence of mucosalis in the oral cavity (Lawson, Rowland and Roberts, 1975). Mucosalis was not recovered from those pigs examined.

In December 1975, on necropsy of a case of lymphosarcoma evidence of PIA was found. This was confirmed histologically and intracellular bacteria with vibrio morphology were demonstrated by silver-staining techniques. It was then decided to try to determine if this was an isolated case or whether PIA did exist as a continuing problem in the herd and was remaining largely undetected. Weekly weights for the pigs after weaning were available and this facilitated the investigation, these being used as the basis for the selection of animals for examination.

MATERIALS AND METHODS

The pigs from the M.D. herd (D) in this study were all presented live and subjected to necropsy immediately after euthanasia and exsanguination. Pathological, bacteriological, immunofluorescent and electron microscopic procedures were carried out as described in Chapter II, with a number of modifications.

Bacteriology

Affected intestinal mucosa from two pigs (483/76 and 20/77), in addition to the usual examination, was treated as described by Love, Love and Edwards (1977). For this one gram of mucosa was added to twenty mls of P.B.S., shaken and then centrifuged to separate the cells. The cells were ground in a Griffith's tube¹ and then plated out on inhibitory and non-inhibitory media.

Immunofluorescence

Cryostat sections were stained using hyperimmune mucosalis anti-sera and F.I.T.C. conjugated G.A.R. Anti-sera to both of the serological variants of mucosalis, i.e., 1248/72 202 and 982/76, were used for this staining. Staining was also carried out using F.I.T.C. conjugated rabbit anti-pig γ -globulin (supplied by Dr. G.H.K. Lawson, Department of Pathology, Edinburgh University Veterinary Field Station). This rabbit anti-pig γ -globulin (R.A.P.) was prepared by the sub-cutaneous injection of pooled pig serum with complete adjuvant into rabbits twice at fourteen day intervals, and then again six months later, nine days before bleeding out.

Selection of Pigs

Weekly weights were available for the pigs from this herd, on entry into the fattening house, and the weekly weight gains

¹ Griffith's tube (homogeniser) - Baird and Tatlock (London) Ltd.

were treated statistically by the cumulative sum technique (I.C.I. Monograph 3, 1964). This technique is used to study sequences of figures which are arranged in the order in which they were derived. It is used to detect changes in the average level of figures, and in determining the point of onset of such changes. It is not appropriate for series exhibiting a steady trend or displaying cyclic variations. The principle applications of cumulative sum methods have, so far, been in industrial quality control, where measurements or counts are made on a process or product at regular intervals and compared with pre-specified levels. The speed with which alterations in average levels are detected is increased by the use of the cumulative sum technique. If the average value of the figures in a series is close to the reference value, some of the differences will be positive and some negative, so that the cumulative sum chart will be horizontal. However, if the average value of the process rises to a new constant level, more of the differences will be positive and the mean chart path will be a straight line sloping upwards. Similarly if the average value of the process falls to a constant level below the reference value, the general slope of the chart will be downwards. Using this technique differences are additive and so accentuated.

The Cumulative Sum, CUSUM or $G_1 = x_1 - K$

where x = weight gain in lbs per week

and K = a constant.

$$G_2 = G_1 + (x_2 - K)$$

$$G_3 = G_2 + (x_3 - K)$$

$$G_n = G_{n-1} + (x_n - K)$$

The calculation works best when CUSUM approximates to zero, therefore the constant is selected as the mean normal value and in this case K was calculated as the mean weight gain for pigs over the fattening period. In this herd the average weight gain over this period was 6.65 lbs per week, so the value of the constant, K, in this study was taken as 6.65.

The first animal to be selected was chosen somewhat arbitrarily. The animal proved to be affected with PIA and on the basis of the weekly weights of this pig a deficit figure of G_n was chosen for selecting pigs for examination. It was decided that pigs which reached a CUSUM of $\bar{15.00}$ or less, would be considered for purchase, and this also applied to pigs which had lost considerable weight from one weekly weighing to the next.

One advantage of the technique is that it is independent of litter mates, which could also be affected.

Histopathological Description - Use of the term villous atrophy

At this point an attempt will be made to define the terms villous atrophy and crypt hyperplasia, as used in these studies. Total villous atrophy will be used to describe a flattened mucosa in which villi were absent. Severe villous atrophy will refer to those cases in which the villi were very attenuated, short and blunt. Villous atrophy describes the mucosa when the villi were shortened and increased in breadth compared to the classical description of the long finger-shaped villus. Other than for the more extreme cases of total and severe, no attempt was made to grade the

severity of the villous atrophy, and neither villus:crypt ratios nor mitotic indices were measured. It is stressed that villous atrophy can occur under a wide variety of circumstances (Arbuckle, 1977), and is only a non-specific, descriptive term. Related to the villous atrophy there could be expected a degree of crypt hyperplasia.

RESULTS

History/Clinical Signs

The first indication of the presence of PIA in the herd since 1965 had been seen at necropsy of a lymphosarcoma pig three months before the commencement of this study. At the time of these observations there were no intercurrent disease problems. Post-weaning colibacillasis had caused trouble in the past but was controlled effectively by a routine immunisation programme. At the beginning of the period a small number of pigs with lymphosarcoma were still present in the herd, but they were transferred to a separate piggery early in the investigation.

The first pig to be examined (pig 110/76) was obtained when the farm manager reported that he had a pig, showing anorexia and failure to gain weight as well as its litter mates, although it was not thin or apparently ill.

Pigs 294/76 and 405/76 were culled for humane reasons. These pigs showed flaccid paralysis and remained in a "dog-sitting" position for long periods. Weekly weights were not available for these animals. Pig 379/76 presented similar clinical signs of flaccid paralysis but had been weighed weekly.

At no time during the study were extremely poor, ill-thriving pigs seen in the M.D. herd. All pigs selected on the basis of the CUSUM value were apparently healthy on clinical examination alone, even pig 483/76 which had lost fifteen pounds in weight over the previous seven days. The weekly weights and CUSUM values for the pigs examined are presented in Table 3b. Table 3c is included as an example, to show the variation in weight gains and cumulative sums in one litter of six piglets in which a confirmed case of PIA (pig 240/76) was examined. The best pig in this litter had a cumulative sum of +37.15, at the same time at which pig 240/76 had a CUSUM of -26.85, and was killed for examination. The other pigs in this litter varied between these two extremes.

Pigs 240/76, 379/76 and 20/77 were selected for examination because their CUSUM values had reached a figure of less than -25.00. Although pig 483/76 had a CUSUM of only -17.60 it had lost fifteen pounds in weight over the previous seven days, and was selected on the combined evidence of CUSUM value and the sudden marked weight loss. Pigs 293/76 and 314/76 were selected at CUSUM values of -18.20 and -16.25 respectively, in an effort to obtain some information on those pigs with relatively poor performances but not sufficiently poor, at the time of selection, to have reached a CUSUM value of -25.00 or thereabouts. The numbers of pigs with CUSUM values below -10.00 are presented in Table 3d.

In those eleven pigs with a CUSUM value of < -20.00 this maximum deficit, i.e., minimum CUSUM value, was reached at an average of $18\frac{1}{2}$ weeks of age, the range being from 14 to 24 weeks of age. For those pigs with a CUSUM value of less than -25.00 which were not

Table 3b Weekly Weights and CUSUMS of Pigs Examined at Necropsy

Pig Number	1		2		3		4		5		6		7		8		9		10		11		
	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	
110/76	64	63	7.65	73	4.30	79	4.95	84	6.60	95	2.25	96	7.90	94	16.55	84	33.20	78	45.85	72	58.50		
240/76	40			49	4.30	52	7.95	63	3.60			71	8.90	70	16.55	71	22.20	73	26.85				
379/76	40	37	9.65	36	17.30			38	28.60	39	34.25												
483/76	51	59	1.35	69	4.70	75	4.05	60	17.60														
20/77	82	99	10.35	98	2.70	100	1.95	103	5.60	102	13.25	108	13.90	114	14.55	104	31.20						
293/76	21	26	1.65	30	4.30	38	2.95	40	7.60	43	11.25	49	11.90	52	15.55	56	18.20						
314/76	32	38	0.65	41	4.30	44	7.95	46	12.60	49	16.25												

W = Weight in lbs. C = CUSUM = weight gain in lbs per week - 6.65 (see page 115)

No weights were available for pigs 294/76 and 405/76.

Table 3c Weekly Weights and CUSUMS in One Litter.

Weeks Since Entered Fattening House --

Pig Number	1		3		4		5		7		8		9		10		11		12	
	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C
3765(240/76)	40		49	4.30	52	7.95	63	3.60	71	8.90	70	16.55	71	22.20	73	26.85				
3766	43		55	1.30	59	3.95	72	2.40	88	5.10	87	2.55	92	4.20	95	7.85	103	6.50	111	5.15
3767	56		70	0.70	76	0.05	90	7.40	106	10.10	111	8.45	124	14.80	134	18.15	146	23.50	157	27.85
3769	59		74	1.70	79	0.05	81	4.60	80	18.90	86	19.55	102	10.22	112	6.85	123	2.50	133	0.85
3770	45		59	0.70	61	3.95	75	3.50	82	2.90	82	9.55	83	15.20	91	13.85	96	15.50	110	8.15
3772	58		75	3.70	80	2.05	95	10.40	118	20.10	127	22.45	143	31.80	154	37.15	162	38.50	177	46.85

Table 3c (Cont'd)

Weeks Since Entered Fattening House --

Pig Number	13		14		15		16		17		18		19		20		
	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	
3766	118	-	4.80	128	1.45	137	0.90	147	4.25	154	4.60	164	7.95	174	11.30	184	14.65
3767	170	+	34.20	178	35.55	193	43.90	200	44.25								
3769	145	+	6.20	160	14.55	173	20.90	184	25.25	192	26.60	203	28.95				
3770	117	-	7.80	131	0.45	139	0.90	150	5.25	159	7.60	172	13.95	185	20.30	199	27.65
3772	190	+	53.20	201	57.55	215	64.90	228	71.25	237	73.60						

Table 3d The Figures for Pigs in Herd D. Fattened During the Period from March 1976 to February 1977.
Numbers of pigs passing through the fattening house = 300.

Lowest Value	CUSUM Reached	Numbers of Pigs	% of Throughput within that range of CUSUM Values	Numbers Examined at Necropsy	Number with PIA at Necropsy
-10.00 to >-15.00		15	5.00	0	-
-15.00 to >-20.00		14	4.67	3	1
-20.00 to >-25.00		1	0.67	0	-
≤-25.00		10	3.00	4	4

killed, there was a negative CUSUM for an average of 18 weeks of the fattening period and this persisted for an average of eleven weeks after the maximum deficit had been reached.

Pathological Findings

The gross and histopathological findings are summarised in Table 3e, and related to the other results. The changes of PIA were, as described in Part A of this chapter. Two of the cases, pigs 240/76 and 20/77, were in fact intermediate cases and showed features of RI as well as of PIA. The two pigs which did not show evidence of PIA, either grossly or microscopically, will be considered in more detail.

Pig 293/76

This pig had an umbilical hernia and the sac was lined by moist granulation tissue and contained omentum. A twenty centimetre length of the serosal surface of the ileum, fifteen centimetres proximal to the ileo-caeco-colic junction, was roughened, due to the presence of blood stained granulation tissue. The intestinal wall in this area was thickened for ten to fifteen centimetres both proximally and distally, but the mucosa appeared normal and much of the thickening was due to oedema and some muscular hypertrophy.

Histologically the mucosa of the terminal small intestine showed villous atrophy and crypt hyperplasia. There was considerable variation in the shape of the villi, and a massive infiltration into the lamina propria of eosinophils. They were present in all areas of the lamina propria and were the only cell type increased in

Table 3e Results of Examination of Pigs from Herd D.

Pig Reference	Reason for Slaughter	Evidence of PIA Macroscopic	Intracellular Vibrios Demonstrated Mod. Z.N. Silver Fluores- E.M. Staining cence	Other Changes
110/76	Anorexia/Failure to Grow (CUSUM -58.50)	+	N.D.	+
240/76	CUSUM -26.85	+	N.D.	+
379/76	CUSUM -34.25 flaccid paralysis	+	+	+
405/76	flaccid paralysis	+	+	+
483/76	CUSUM -17.60 loss of 15 lbs in wt. in seven days.	+	+	+
20/77	CUSUM -31.20	+	+	+
294/76	flaccid paralysis	-	N.D.	N.D.
293/76	CUSUM -18.20	-	N.D.	-
314/76	CUSUM -16.25	-	N.D.	-

N.D. = Not Done.

glomerulonephritis
cerebrospinal
angiopathy.
grossly-thickening
of terminal ileum.

number, the other cells being present in normal, low numbers. In the mid small intestine and large intestine there were some eosinophils in the lamina propria, but this was not as marked as in the ileum. No evidence of parasites were seen. The lymphoid follicles of Peyer's patches showed a moderate hyperplasia. The oedema and muscular hypertrophy seen grossly were confirmed histologically. The serosa was thickened due to the presence of granulation tissue, in which only very occasional inflammatory cells were present, although in some areas it was markedly congested.

Pig 314/76

There were no gross lesions in the alimentary tract of this pig. Histologically in the ileum there was a severe villous atrophy and an associated crypt hyperplasia, and in some areas the villi were present only as small ridges in the mucosa. There was a massive infiltration of eosinophils into the lamina propria and these were seen in close proximity to, and also within the epithelium. Other than for the eosinophilic infiltration the cells in the lamina propria were few in number. There was oedema of the lamina propria. In the mid small intestine there was villous atrophy and crypt hyperplasia, but this was not as severe as in the terminal ileum. The cells in the lamina propria were increased in number and included some eosinophils, but the predominant cell type was the plasma cell. The epithelium of the large intestine appeared normal, with a substantial mucus-secreting cell population. The lamina propria was oedematous in some areas, and there was an increase in the cellularity, the majority of the cells being plasma cells, with only occasional eosinophils present.

Pigs with evidence of PIA

In all the pigs with changes of PIA, histologically there was evidence that they were at the recovery stage and that resolution of the intestinal lesion was underway. This will not be considered further at this time, as it is, along with the description of the ultrastructure, the subject of Chapter IX, and the description there is appropriate for these cases.

Electron Microscopy

In those cases in which bacteria were demonstrated intracellularly (Table 3e) the electron microscopic appearance was as described for the cases of PIA in Part A of this chapter. The bacteria were seen as curved osmiophilic bodies free within the apical cytoplasm of the epithelial cells and not surrounded by host cell membranes. The bacteria could not be distinguished from those cases of PIA from other sources.

As well as free within the apical cytoplasm of epithelial cells, occasionally collections of bacteria were included in a membrane-bound vesicle. Also bacteria, resembling those seen in the epithelium, were observed within phagolysosomes in macrophages and neutrophils in the lamina propria, the epithelium and the gland lumen.

Bacteriological Results

In those cases in which modified Z.N. stained impression smears of the mucosa were made (Table 3e), acid-fast bacteria were seen within epithelial cells. Also free acid-fast vibrios were present either in clumps or singly.

Mucosalis was not recovered from any of these pigs. The primary plates were not overgrown with contaminants and at the lower dilutions colonies of mucosalis, if present, should have been clearly visible. In fact in many cases the washed mucosa was shown to be remarkably clean on plating out and incubation. C. coli was isolated from some of these pigs. These isolates were catalase positive, and were not agglutinated in slide agglutination tests using either 982/76 or 1248/72 2C2 mucosalis anti-sera.

Using the method of Love et al. (1977) on tissues from pigs 483/76 and 20/77 did not result in the isolation of any bacteria resembling mucosalis.

Immunofluorescence Results

Cryostat sections were stained using either standard mucosalis anti-serum (1248/72 2C2) or 982/76 mucosalis anti-serum. The latter was used since the pig unit (herd K) established with stock from herd D had yielded cases of PIA and NE from which this serological variant of mucosalis had been recovered. A number of cases were also stained using crude F.I.T.C. conjugated rabbit anti-pig γ - globulin (R.A.P.).

With pig 110/76 there was some fluorescence in the apical cytoplasm of affected epithelial cells using either 1248/72 2C2 or 982/76 mucosalis anti-sera. However, this fluorescence was more diffuse and less particulate than that usually seen in PIA cases. In the other cases of PIA there was a variable amount of specific particulate fluorescence in the apical cytoplasm of affected epithelial cells, when stained with either 1248/72 2C2 or 982/76 mucosalis

anti-sera. The amount of particulate fluorescence in these cases was not usually as great as in the positive controls stained in parallel, or as expected from the numbers of organisms shown to be present by silver-staining and electron microscopy. In these cases there were also cells, probably macrophages, with intracytoplasmic specific particulate fluorescence, present in the lamina propria, within the gland lumen, and within the epithelium. These are considered to be a feature of the recovery phase of PIA and are dealt with in greater detail in Chapter IX.

In sections stained with crude R.A.P. there was positive fluorescence, some of which was particulate and some more diffuse in nature, in the apical cytoplasm of the epithelial cells. This fluorescence corresponded to those areas fluorescing when stained with mucosalis anti-sera. In these same sections, stained with R.A.P., there was also positive particulate fluorescence in those cells described above as probably being macrophages and which showed specific particulate fluorescence with mucosalis anti-sera. In cryostat sections of normal intestine stained in parallel with R.A.P. there was often some diffuse fluorescence especially in the apical region of the crypt cells, but this was never as bright as that described in these PIA cases. This is another feature of the recovery phase of PIA, considered more fully in Chapter IX.

DISCUSSION

A number of interesting points arose from this study. There were seven confirmed cases of PIA, representing $2\frac{1}{2}\%$ of the throughput over the twelve month period of study. Seven further pigs had CUSUM values of below -20.00, and all the pigs with values below this level which were necropsied had changes of PIA. This means that in addition to the seven confirmed cases of PIA there were a further seven probable cases. Even this figure is at best only a conservative estimate and does not include pigs 293/76 and 314/76, which had intestinal changes, but no evidence of PIA. Of the pigs in the CUSUM range -15.00 to -20.00 three were examined and one of these had PIA, the other two were pigs 293/76 and 314/76. The other pigs falling into this CUSUM range are not included in the estimate of the incidence of PIA. So these figures only encompass the worst pigs in the fattening period and no attempt is made to evaluate to what extent PIA is responsible for the slightly better growing, but still poor, pigs.

At the present state of knowledge of PIA it is important not to extrapolate too far from the known facts, but it is of interest to consider the two pigs 293/76 and 314/76. The gross findings in pig 293/76 were similar to those of PIA and had caused a similar change in the performance figures of this pig to those of pigs with PIA. The changes may have been related to the umbilical hernia in this pig and the presence of the thickened segment of bowel within the hernial sac at some stage. However, the segment of intestine involved was part of the terminal ileum, which is relatively immobile, having a shorter mesentry compared to the more proximal

small intestine, so that it would appear less likely for this segment to enter the hernial sac. The changes in this pig closely resembled those seen in pig 355/76 (Chapter III, Part A). Histologically in both cases there was a massive eosinophil infiltration into the lamina propria of the terminal small intestine. In pig 355/76 there were occasional adenomatous glands present, and mucosalis was recovered from the mucosa. In both pigs the lesions were present in the terminal ileum, involving the area in which lesions are seen in the majority of PIA cases, and there was no evidence of helminth parasites. Eosinophils are reputedly attracted to antigen-antibody complexes (Kay, 1976; Beeson, 1977), and the possibility exists that these two cases were in the recovery phase of PIA, with antigen-antibody complexes being formed within the epithelium. This can only be speculative in our present state of knowledge of the function of the eosinophil and understanding of PIA. A similar explanation could be put forward for the changes in pig 314/76 as again there was a massive infiltration of eosinophils into the lamina propria, and a close association between the eosinophils and the epithelium. If eosinophils were infiltrating in response to antigen-antibody complexes, this might explain the histological findings of villous atrophy and crypt hyperplasia. These latter changes are seen in coeliac disease, gluten enteropathy, in which an immunological response to gliadin of the wheat protein gluten is responsible for the intestinal pathological changes (Ferguson, 1976). Again in pig 314/76 the eosinophilic infiltration was into the region of the intestine commonly involved in PIA.

The fact that mucosalis was not recovered even from the pigs

with gross lesions of PIA in this study and the uncertainty as to whether this was due to a host-cell dependence of the organism (see below) makes it difficult to assess the failure to recover mucosalis from pigs 293/76 and 314/76. Villous atrophy and crypt hyperplasia have been described in cases of "three week scours" from which mucosalis has been isolated (Chapter III, Part D), and also during the recovery phase of experimental cases of PIA (Chapter VII). Eosinophil infiltration into the lamina propria of recovery phase PIA cases may also be seen occasionally. However, since there was no definite evidence that these were cases of PIA, or that the pigs had been infected with mucosalis, they were not included in the figures of the incidence of PIA in the herd, and pigs with as poor performance figures were similarly not included.

The absence of notable clinical signs in a number of these pigs is interesting. In many herds PIA is a condition of the post-weaned pig, in which severe wasting is characteristically seen (Rowland and Rowntree, 1972; Martinsson et al., 1974; Part A, of this chapter). However, a number of workers describe cases in abattoir material, in which animals there has been no observed clinical evidence of disease (Emsbo, 1951; and others). The recovery of affected pigs reported by others (Rowland and Rowntree, 1972; Rowland and Lawson, 1974) is also supported by this study. The pigs which had performance figures as poor as those of pigs with PIA, proceeded to bacon weight and were slaughtered. The absence of intercurrent disease in this herd, adds weight to the probability that these pigs were affected with PIA, the lymphosarcoma pigs having been removed from the herd and the colibacillosis problem being effectively controlled.

The economic cost of this will be obvious in that pigs will have to be kept longer to reach slaughter weight. Poor conversion figures during the period of infection, will result in an increased cost of fattening. Although not a problem in this herd, the loss from the death of pigs with necrotic enteritis could also contribute to the economic loss. The figures available did not allow an exact assessment of the losses, and this was not the primary objective of the study. As only the worst pigs have been considered the true effect on the herd performance is likely to be greater than indicated by the occurrence of confirmed cases of PIA.

The failure to recover mucosalis bacteriologically from these pigs is intriguing. An organism closely resembling mucosalis was demonstrated by a number of other techniques; modified Z.N. stained impression smears, in silver stained sections, by immunofluorescence and ultrastructurally. There are a number of possible reasons for this failure to isolate mucosalis. There was evidence that these cases were at the recovery phase of PIA, both histologically and ultrastructurally. Immunofluorescent studies also suggested that the bacteria may have had antibody bound to them. If these findings are correct it may be that the bacteria were damaged sufficiently to prevent recovery, or if there was antibody present on their surface they may not have been able to grow. Another possibility is that the bacteria involved in this M.D. herd have become host cell dependent and require factors not usually required by those mucosalis strains already isolated and described. This factor(s) may not have been present in the media on which isolation was attempted. It is interesting that mucosalis has been isolated from pigs in the unit established with stock from this herd. If the organism is the same in

both herds then this is evidence against host cell dependence. The immunofluorescent results were unequivocal in resolving this and positive fluorescence was seen after staining with either 1248/72 2C2 or 982/76 mucosalis anti-sera. It could be that a previously undescribed variant of mucosalis is involved in this herd. It is of interest that in cases of PIA showing evidence of recovery, from another herd and in experimental cases during the recovery phase, mucosalis could sometimes be recovered, although often in reduced numbers (Chapter IX). Finally, the possibility cannot be excluded that the organism involved in this herd was different from those isolated previously from cases of PIA from elsewhere. Certainly no other bacteria were recovered in large numbers from these cases.

Three of the pigs examined and found to have evidence of PIA were presented with posterior ataxia and flaccid paralysis. The significance of these findings is unknown. In one of these pigs there was a cerebrospinal angiopathy, no changes to account for the clinical signs were seen in the other two.

In conclusion, PIA was shown to be present in this herd at a level involving $2\frac{1}{2}\%$ of the throughput of pigs (confirmed) and probably at least as high as 5% of the throughput. Without careful monitoring of the herd's performance the condition could very easily remain undetected. Further work will be necessary to assess fully the occurrence of the disease and its true economic significance.

PART D. OBSERVATIONS ON "THREE WEEK SCOURS" IN PIGLETS, WITH
REFERENCE TO CAMPYLOBACTER SPUTORUM SUBSP. MUCOSALIS

INTRODUCTION

Diarrhoea in suckling piglets of two to four weeks of age is widely reported in the United Kingdom under a wide variety of names; "non-infective diarrhoea" (Smith and Jones, 1963; Jones, 1967a), "milk-scours" (Stevens, 1963), "nutritional scours" (Jones, 1967a), and "three week enteritis" (Stevens, 1963). In Holland, "white scours" (Mouwen, 1972) occurring at three weeks of age is recognised. From the terminology it can be seen that the widely held view is that the condition is not of infectious aetiology and is related to nutritional changes, such as the introduction of creep or alterations in the sow's milk. Stevens (1963) suggested that E. coli may be involved. A detailed study of "white scours" has been reported by Mouwen (1972). He proposed the following pathogenesis; increased food intake and/or gastrointestinal hypomotility leading to dysbacteriosis with proliferation of E. coli in the increased intestinal contents, the E. coli producing mucosal damage with resultant malabsorption and steatorrhoea. The histological lesions were villous atrophy, crypt lengthening and increased lymphoid infiltration into the lamina propria. These changes are considered non-specific by Arbuckle (1977) and they occur in a number of conditions in the pig; TGE (Hooper and Haelterman, 1966), E. coli infection (Moon, Nielsen and Kramer, 1970), salmonella cholerae-suis infection (Arbuckle, 1975), in healthy pigs (Kenworthy and Allen, 1966; Kenworthy, 1971) and in pigs fed a protein-

calorie deficient diet (Platt, Heard and Stewart, 1964).

Rotaviruses have recently been isolated from diarrhoeic animals of many species (Anon. 1975), including pigs (Woode et al., 1976; McNulty et al., 1976, and others). Isolations have been made from diarrhoeic piglets both before and after weaning. Their true significance has not yet been defined but pig, human and calf viruses all produce diarrhoea and intestinal lesions in gnotobiotic piglets. The role they play, if any, in diarrhoea in unweaned piglets has yet to be investigated.

In pigs fed on rations containing soya protein intestinal changes have been reported, due to an immune response to soya protein constituents (Porter, 1977). The role which this plays in diarrhoea in piglets at three weeks of age, a time when more solid food is taken, has not been investigated. It has been suggested by Ferguson and Jarret (1975) that villous atrophy occurs due to a T-cell mediated reaction in the intestine, such as occurs after allograft reaction of small intestine (Ferguson and Parrott, 1973). This is consistent with Arbuckle's hypothesis that the lesion is non-specific, as presumably a variety of infectious or dietary antigens could invoke such a response.

During the course of this study the opportunity arose to study piglets from an outbreak of diarrhoea in three week old piglets, on a farm in which PIA exists as a continuing endemic problem in older pigs.

MATERIALS AND METHODS

The piglets studied all originated from one herd (B). All of these were presented for necropsy, at the routine pathology

diagnostic service offered by the department, except for pig 298/75, which was presented live on request.

Necropsies were carried out, tissues for bacteriology, histopathology and electron microscopy (pig 298/75 only) were taken and processed as described in Chapter II.

RESULTS

History/Clinical Signs

In the closed Large White herd (B) from which the piglets originated various feeding trials, growth trials and management experiments are carried out. For a number of years PIA has been present in this herd, and mucosalis has been consistently isolated from affected pigs. Spirochaetosis has been a problem in the post-weaned pool of pigs, the organisms isolated to date have not resembled Treponema hyodysenteriae (D. Taylor, personal communication, 1977). Previous problems in the farrowing house have included failure of the piglets to consume creep feed. In more recent months piglets of two to three weeks of age had shown evidence of diarrhoea and failure to thrive. Response to antibacterial therapy had been variable, and many of the pigs remained in poor condition for a period of time although few animals actually died. Two of the pigs examined were older, five and nine weeks old, but were still housed within the farrowing house at the time of death. Normally after weaning at five weeks of age, the pigs were moved to covered outside accommodation. Occasional pigs were kept in the farrowing house for some time after weaning (e.g. pig 210/75) if they were poorly grown. It was from the post-weaned pool of pigs in the outside accommodation that cases of PIA were normally presented.

Details of the pigs examined are presented in Table 3f.

Table 3f Piglets Examined from Farrowing House.

Piglet Reference	Age	Clinical Signs
210/75	9 weeks	Scouring, ill-thrift, death.
287/75	15 days	Scouring, poor-growth, death.
293/75	21 days	Scouring, ill-thrift, death.
294/75	21 days	Scouring, ill-thrift, death.
298/75	21 days	Presented live, scouring, poor growth.
M.1353/75	21 days	Diarrhoea, poor growth.
335/75	21 days	Diarrhoea, poor growth.
336/75	5 weeks	Diarrhoea, poor growth.

The pathological, histopathological and bacteriological findings in these pigs are presented in Table 3g. The changes in the alimentary tract are then considered in more detail in the appropriate sections.

Pathology

Gross findings

Pig 210/75. An emaciated poorly grown carcass, with lesions restricted to the alimentary tract. There were numerous erosions and foci of congestion in the gastric mucosa. The terminal fifty

Table 3g Pathological and Bacteriological Results

Pig Reference	Gross Pathology	Histopathology	Bacteriology
210/75	Emaciation, Possible thickening of T.S.I. and L.I.	PIA	+ve <u>Mucosalis</u> L.I.
287/75	Poorly grown	Villous Atrophy Crypt Hyperplasia	-ve.
293/75	Necrotising Enteritis, T.S.I. Fibrinous Peritonitis, Pneumonia.	Necrotising Enteritis ex- tending to the serosal surface.	+ve. <u>Mucosalis</u> T.S.I.
294/75	Necrotising Enteritis, M.S.I. Peritonitis.	Necrotising Enteritis ex- tending to the Serosal surface.	N.D.
298/75	N.A.D.	Villous Atrophy Crypt Hyperplasia	+ve. <u>Mucosalis</u> T.S.I. and L.I.
M.1353/75	N.A.D.	N.D.	-ve.
335/75	N.A.D.	Villous Atrophy Crypt Hyperplasia	-ve.
336/75	N.A.D.	Villous Atrophy Crypt Hyperplasia	-ve.

N.D. = Not done

N.A.D.= No abnormalities detected.

centimetres of the small intestine, the caecum and proximal twenty centimetres of the colon were thought to be thickened, but the thickening was not marked and it was not possible to be certain on the gross appearance. The surface of the mucosa of both the small and large intestines was smooth and Peyer's patches in the ileum were easily distinguished.

Pig 293/75. A small, emaciated carcass. The ventral dependent areas of the apical and cardiac lobes of both the left and right lungs were firm, consolidated and pneumonic. There was a fibrinous peritonitis, with the abdominal cavity full of blood-stained fluid. The distal one third of the ileum was friable, thickened, dark red in colour and the mucosa had undergone a coagulative necrosis with the inflammatory process extending through the wall to the serosal surface.

Pig 294/75. This piglet was in very poor bodily condition and had an extensive fibrinous peritonitis. There was a massive primarily coagulative necrosis of the mucosa of the middle one third of the small intestine, extending into the deeper layers of the wall.

Pigs 287/75, 298/75, M.1353/75, ~~335/75~~ and 336/75. These five piglets will be considered collectively as the findings in all five were similar. All were in poor bodily condition, and at necropsy no gross lesions were found except for a single congenital polycystic kidney in pig 335/75, the other kidney was normal and there was no evidence of renal failure. No gross abnormalities in the mucosa of the alimentary tract were seen.

Histopathology

Pig 210/75. The mucosa was not increased in width to any extent, but the normal architecture was replaced by proliferating adenomatous glands in the terminal ileum. Bacteria of vibrio morphology were demonstrated in the apical cytoplasm of the affected epithelial cells in silver stained sections.

In the large intestine there was considerable variation in gland shape and size. Some glands were definitely adenomatous and lined by immature, proliferating epithelial cells. Other glands of a very small diameter were lined by a flattened epithelium, and there were also some glands with a wide diameter lumen, lined by only a flattened, attenuated epithelium. Mucus-secreting cells were not a prominent feature. In other areas the adenomatous glands were not seen, and the epithelium lining the glands was flattened. In the lumina of many of these glands there appeared to be mucus, but only a few of the epithelial cells stained for mucin. The impression gained was that there had been a massive outpouring of mucus, leaving a flattened, "exhausted" epithelium.

Pigs 293/75 and 294/75. There was an extensive, primarily coagulative necrosis of the mucosa with extension of the destruction and inflammatory process to involve all layers of the wall. Remnants of glandular epithelium were visible, but it was not possible to identify whether or not these had previously shown adenomatous change.

Pigs 287/75, 298/75, 335/75 and 336/75. The changes in these pigs were essentially similar, but demonstrated best in pig 298/75, which was submitted live for examination. In the other pigs

there was some post-mortem degeneration.

In the terminal ileum there was villous atrophy and crypt hyperplasia. The crypts were lengthened, showed increased mitotic activity and occasionally had a crowded appearance, without being definitely adenomatous (Figure 53). In most areas few goblet cells were present. In the lumina of some glands there was cellular debris and occasional neutrophil polymorphs. Silver-staining failed to conclusively reveal the presence of intracellular bacteria, although occasional possible bacterial forms were seen.

In the large intestine the crypts were lengthened and there was an increase in the number of mitoses. There was also an increase in the number of cells in the lamina propria, and some glands contained inflammatory cell debris (Figure 54). The findings in silver-stained sections of the large intestine were, as for the small intestine, inconclusive.

Bacteriological Results

As can be seen from Table 3g, mucosalis was recovered from pigs 210/75, 293/75 and 298/75. The numbers isolated were as follows:

Pig	Numbers of viable <u>mucosalis</u> per gram wet weight of mucosa
210/75 L.I.	3.04×10^5
293/75 T.S.I.	$\geq 3.52 \times 10^4$
298/75 T.S.I.	8×10^2
298/75 L.I.	$\geq 8 \times 10^2$

Mucosalis was not recovered from the other piglets (294/75 was not examined bacteriologically). These piglets had been presented dead, and in some cases the plates were overgrown with other members of the gut flora.

Gram-stained smears of small and large intestinal contents were examined, and also wet-preparations under phase for spirochaetes. No spirochaetes were seen. Cultural examination for enteropathogenic E. coli and salmonella spp. was negative, and no other bacteria, which were considered to be of significance, were seen or isolated.

Electron Microscopy

Tissues from the terminal ileum and large intestine of pig 298/75 were examined ultrastructurally. Bacteria were not seen within the epithelial cells, as has been described for mucosalis in PIA. Ultrastructural alterations in the cells were not noted. In the lumina of occasional crypts a variety of bacteria were seen, none of which resembled mucosalis.

DISCUSSION

This would appear to be the first report of the isolation of mucosalis from the intestinal mucosa of naturally diseased pigs in which there was not evidence of adenomatous change. Lawson and Rowland (1974) reported that they had been unable to isolate mucosalis from the intestines of normal pigs, or of pigs with enteropathies in which there was not an adenomatous component. Lawson, Rowland and Roberts (1975) did however describe the isolation of mucosalis from the oral cavity of piglets which did not have evidence of PIA.

The histological changes of villous atrophy and crypt hyperplasia which were seen, are those described for "three week scours". However, these changes are seen under a variety of circumstances and are considered to be non-specific. It may be that mucosalis should be added to the list of possible aetiological agents of such a change.

In the experimental work reported in subsequent chapters occasional piglets which had been orally dosed neonatally with a suspension of mucosalis or of adenomatous mucosa, grew poorly, had diarrhoea and at necropsy had changes of villous atrophy and crypt hyperplasia, as described here. Mucosalis was isolated in low numbers from some of these piglets. In the successful transmission experiments reported in Chapter VII, piglets killed before developing gross evidence of PIA showed histological changes of villous atrophy and crypt hyperplasia in the small intestine.

From the history of the herd and the changes of PIA present in pig 210/75, it is tempting to speculate that villous atrophy and crypt hyperplasia are pre-adenomatous changes and that under certain circumstances these may present clinically as diarrhoea in suckling piglets. The isolation of mucosalis from pigs with these changes, in this herd and the experimental work referred to above, provide support for this hypothesis. However, the possibility of other aetiological agents being involved cannot be dismissed; virological examination was not undertaken, and immune or dietary factors were not investigated. No other significant bacterial agents were, however, seen or isolated.

In the piglet the period of three to four weeks of age is described as one of critical antibody deficiency (Brown et al., 1961; Miller et al., 1962). At this age the level of colostrum-acquired antibodies is low and the active production of antibodies has not yet reached a level indicative of immunity. The significance of this in relation to "three week scours" is unknown at present.

The failure to demonstrate bacteria within the epithelial cells of affected tissue by electron microscopy may be due to the low numbers present. Only a very small amount of tissue can be examined by this technique. Silver-stained sections are also difficult to interpret when organisms are scarce. This problem was encountered in the experimental infections with mucosalis, in which again only small numbers of mucosalis were recovered from the mucosa. So whether or not mucosalis was present intracellularly in these cases remains unresolved.

Pig 210/75 is of interest for a number of reasons. The intestine was only very marginally thickened to the naked eye and unless histology had been undertaken the changes would have remained undetected. In fact, histologically, the whole mucosa showed frank adenomatous change. The appearance of mucus depletion in the large intestine was unusual. Immune-complexes have been described as stimulating mucus release from goblet cells (Bloch and Walker, 1977). The presence of this pig, with changes of PIA, still within the farrowing house would provide a source of infection for the other piglets housed there. Since piglets with PIA are often, like this one, in poor bodily condition and small for their age, there may be a tendency

to accommodate them with the younger pigs rather than putting them into the weaner pool. This practice is obviously not a sound one and such pigs are better kept isolated from the rest of the herd.

These preliminary observations on "three week scours" in a herd with PIA suggest that mucosalis should be considered in such cases and examination of other pigs in the herd for PIA or other proliferative enteropathies might prove rewarding. Further work is, however, necessary to clarify the situation. The need for continuing investigations into diarrhoea in the suckling piglet is once again emphasised.

SECTION A

(CHAPTERS IV, V and VI)

THE INFECTIVITY OF *CAMPYLOBACTER SPUTORUM*

subsp. *MUCOSALIS* FOR THE PIG

SECTION A

INTRODUCTION TO INFECTIVITY EXPERIMENTS

It would seem to be appropriate at this point to discuss the factors affecting the establishment of bacterial enteric disease. Experimentally it is often necessary to expose animals to artificially large numbers of bacteria, and even this apparently abnormal dose, by itself, may be insufficient with certain bacteria to produce disease. Consequently, resort has to be made to modifying the host's defence mechanisms in order that infection may be satisfactorily established. Much work has been carried out with human enteric pathogens in laboratory animals, and these studies have clarified some of the factors controlling the establishment of enteric infections.

Gastric acidity is one of the first defence mechanisms which play a part in destroying orally administered enteric pathogens. With Vibrio cholerae orally administered to human volunteers the number of organisms required to produce diarrhoea is reduced and the diarrhoea is of greater severity where the gastric acidity is neutralised (Hornick et al., 1971). Reduced gastric acidity also seems to increase the susceptibility to salmonellosis (Giannella, Broitman and Zamcheck, 1971). The effect of gastric acidity can be neutralised in the experimental situation by either orally administered alkali, as with calcium carbonate in the canine model of cholera (Sack and Carpenter, 1966), or bypassing the stomach with direct inoculation of the organisms into the intestine (De and Chatterje,

1953). Gastric secretions have been investigated for bactericidal mechanisms other than the low pH, and in the suckling rabbit the stomach contains a factor which converts ingested milk into a bactericidal substance (Smith, 1966). This latter species is, however, abnormal in having low or zero counts of bacteria in the upper alimentary canal. Gastric mucus, however, protects bacteria (Grady and Keusch, 1971). In their experiments with Vibrio coli James and Doyle (1947) administered the bacteria with mucus in attempts to demonstrate pathogenicity. Under certain circumstances organisms pass quickly through the stomach, this takes place if the stomach is full and the organisms are present in fluid (Mossel and Oei, 1975). This substantially reduces the dose necessary to produce infection.

The importance of peristalsis and normal motility of the bowel in clearance of introduced bacteria has been established (Dixon, 1960; Abrams and Bishop, 1966 and 1967) and the reduction in peristalsis produced by opiates has been widely used in experimental work in this field. As early as 1885 Koch used opium to slow gastrointestinal peristalsis in work with V. cholerae in the guinea pig, and Burrows, Elliott and Havens (1947) used a similar model. Such treatment has been used in salmonella infections in guinea pigs (Kent, Formal and Labrec, 1966), in shigella infection in the guinea pig (Formal et al., 1963) and in V. cholerae in adult mice (Knop and Rowley, 1975a). Peristalsis has been shown in a number of models to be one of the important factors responsible for elimination of introduced organisms from the gut, and it has been suggested that

rapid peristalsis quickly results in removal of the organisms from the small intestine and into the large intestine, where they are inhibited by other mechanisms (Dixon, 1960; Miller and Bohnhoff, 1962).

The resident flora has a protective effect through its influence on gastro-intestinal peristalsis (Abrams and Bishop, 1967; Abrams, 1970), the presence of the indigenous flora being responsible for the "normal" propulsive activity to a significant degree. It is also effective through its antagonism of introduced enteric pathogens (Savage and McAllister, 1970; Savage, 1972). Maintenance of a low oxidation-reduction potential and production of volatile fatty acids by the resident flora have both been implicated in this (Meynell, 1963). Competition between pathogen and resident flora for available nutrients has also been suggested (Hentges and Freter, 1962). This inhibition of the introduced organism by the resident flora can be overcome by reduction or temporary removal of the flora using antibacterial drugs. Such antibacterial treatment was used to establish experimental enteric vibrio and shigella infections in mice and guinea pigs (Freter, 1956), and to increase the susceptibility of mice to Salmonella enteritidis; when mice were pretreated with streptomycin the infective dose of salmonella was reduced from one million to less than ten organisms (Bohnhoff and Miller, 1962). The inhibitory effect of the resident flora may be removed by the use of gnotobiotic animals. In shigella infections in guinea pigs (Formal et al., 1961) such gnotobiotic animals do not require the pretreatment necessary in conventional animals. Experimental enteric infection with E. coli in neonatal gnotobiotic piglets

has been described by Staley, Jones and Corley (1969a). V. coli infection in gnotobiotic pigs has been reported (Andress, Barnum and Thomson, 1968; Kashiwazaki, Namioka and Yabiki, 1971). The problems with and advantages of gnotobiotic experimental animals are highlighted by the relationship between Treponema hyodysenteriae and swine dysentery (Meyer, Simon and Byerly, 1974a, 1974b and 1975). Although the role of the spirochaete in this condition is widely accepted, studies suggest that an interaction between other members of the Gram negative obligate anaerobic flora and the spirochaete are necessary for the production of disease. So although T. hyodysenteriae will colonise the large intestine of gnotobiotic swine disease is not produced unless certain other bacteria are present (Meyer et al., 1974 and 1975; Brandenburg et al., 1977).

Starvation has been used widely in experimental work to increase susceptibility to enteric infection. This has been described for V. cholerae (Goldstein, Merrill and Sprinz, 1966) shigella (Formal et al., 1958) and salmonella (Miller and Bohnhoff, 1962). Starvation is possibly effective through a reduction of gastric secretion (Grady and Keusch, 1971), and a decrease in the antibacterial mechanism which operates in the small intestine (Knop and Rowley, 1975a). In view of this it is interesting that Freter (1974) reported that secretory IgA was only detected in the intestinal secretions of human volunteers, if the samples were collected shortly after a meal. Food and water deprivation, i.e., dietary stress has an effect on the gastro-intestinal microbial flora (Tannock and Savage, 1974). Alteration of diet can also bring about changes in the microbial flora (Mickleson and Klipstein, 1975) and alter the

susceptibility to infection (Dubos and Schaedler, 1962). The presence of intestinal helminths can also influence enteric bacterial infection (Collins, Boros and Warren, 1972; Cypess et al., 1974).

The important role of peristalsis in removing introduced bacteria from the alimentary tract has already been described. One of the features of certain enteropathogenic bacteria is their ability to attach to the intestinal epithelium through specific receptors so overcoming the mechanical removal by peristalsis. The adherence of certain enteropathogenic E. coli in neonatal pigs mediated by the K88 antigen (Jones and Rutter, 1972) is a specific example of this property and vaccination against this antigen affords protection (Rutter and Jones, 1973). Innate resistance in some strains of pigs is related to the absence of the receptor for this antigen on the intestinal epithelial cell (Sellwood et al., 1974). Attachment of V. cholerae to the intestinal epithelium has also been described (Jones, Abrams and Freter, 1976; Jones and Freter, 1976; Freter and Jones, 1976; Nelson, Clements and Finkelstein, 1976). The importance of attachment on other mucous membranes is also recognised, e.g., the respiratory system and mycoplasma infection (Collier and Clyde, 1972) and the genital system and gonococcal infection (Ward and Watt, 1972).

Immune mechanisms operate in the alimentary tract. A local secretory immune system (Tomasi, 1976) operates on mucous membranes including the gut. Such local immunity involves secretory IgA (Tomasi and Grey, 1972; Lamm, 1976) predominantly, and to a lesser extent IgM, although more recently the importance of IgM in

the early neonatal period has been recognised (Allen and Porter, 1977). IgM cells form the majority of immunoglobulin cells in the lamina propria of the small bowel in suckling pigs up to four weeks of age, but in the weaned animal the proportion of IgM cells gradually declines with maturity until by the time the pigs are twelve weeks old ninety per cent of the cells contain IgA (Allen and Porter, 1977). Inhibition of attachment of organisms to the mucosal epithelium is reported to be the method by which the system operates (Freter, 1969; Williams and Gibbons, 1972), and it correlates with the degree of bacterial agglutination within the intestinal lumen (Bellamy et al., 1975). Such agglutination of bacteria within the lumen allows increased removal from the small intestine (Knop and Rowley, 1975c) and also there is an increased antibacterial activity against those remaining (Knop and Bellamy, 1976). This effect has been extensively studied in V. cholerae infection where prevention of adherence of the vibrios to the intestinal epithelium occurs (Freter, 1969; Schrank and Verwey, 1976) through antibody cross-linking of the vibrio population in the lumen (Steele, Chaicumpa and Rowley, 1975), or occupying specific receptor sites. There is also a suggestion that the local immune system may be bactericidal (Chaicumpa and Rowley, 1972), but the capability of secretory IgA in this activity is still unconfirmed (Tomasi, 1976). Shedlofsky and Freter (1974) recently showed how synergism between the ecological and immunological control mechanisms might operate. In their work with V. cholerae the local antibacterial immunity was considerably more effective when operating in conjunction with bacterial

antagonism. They suggest that bacterial antagonism is more important in controlling the lumen flora, whilst the local anti-bacterial immunity operates against invading mucosal associated organisms. The latter is only successful if the numbers of organisms are kept small by other mechanisms such as bacterial antagonism and peristalsis.

From this brief discussion it will be realised that the establishment of enteric infections and experimental disease is not easy, and the factors involved in such establishment are varied and complex. In this section experiments are described in which infection of pigs with C. sputorum subspp. mucosalis was produced. From these studies it was hoped to obtain information about the host-bacterial relationship between the pig and the bacterium associated with PIA, C. sputorum subspp. mucosalis.

CHAPTER IV

INFECTIVITY EXPERIMENTS USING NEONATAL PIGSINTRODUCTION

The neonatal pig was used in these infectivity experiments for a number of reasons. The newborn pig is devoid of immunoglobulins, relies on colostrum as the sole source of serum antibody, and depends on milk for its intestinal antibody during most of the post-natal period (Bourne, 1973). Modifications of the intestinal epithelial cell of the neonatal pig have been described (Staley, 1969; Hardy, Hockaday and Tapp, 1971) which are involved in the non-selective absorption of high molecular weight substances from the luminal surface (Lecce, 1966; Payne and Marsh, 1962). In neonatal rats the intestinal epithelium has been shown to internalize adenovirus by way of these apical tubules and vesicles (Worthington and Graney, 1973a and 1973b), and in the neonatal pig entry of TGE virus into the intestinal epithelial cells is through this network (Wagner, Beamer and Ristic, 1973). Uptake of E. coli by neonatal intestinal epithelium in the pig has been described (Staley, Jones and Corley, 1969d). TGE is, however, infective after the disappearance of these apical tubular structures, but the reduced number of virus particles gaining access to the cell may be related to the disappearance or marked reduction in the development of the apical tubular system. Kenworthy, Stubbs and Syme (1967) described the occasional presence of these tubules and vesicles in older pigs.

An age related resistance has been described for a number of conditions. In porcine TGE, although all ages are susceptible, the frequency, severity and duration of diarrhoea is greater in new born pigs (Moon, Norman and Lambert, 1973). In reovirus-like agent associated diarrhoea, the neonatal pig is more susceptible, and susceptibility declines with age (Lecce, King and Mock, 1976). For vibrio cholerae, resistance to infection in mice (Chaicumpa and Rowley, 1972) and rabbits (Burrows and Sack, 1974) is age related, in that the neonatal animal is susceptible to challenge and this susceptibility is lost by ten to fourteen days of age.

Specific colostral antibody can also affect enteric infection by orally administered pathogens, and such a protective effect has been described for TGE virus (Bohl and Saif, 1975) and E. coli (Rutter and Jones, 1973; Rutter et al., 1976) in piglets. This is reputedly related to interference with adherence of the organism with the epithelial cell. If such association and/or attachment is a necessary prerequisite for the entry or uptake of mucosalis into the intestinal epithelium, colostrum may influence the establishment of such infection. In these experiments an attempt was made to ascertain the influence of colostrum on the establishment of mucosalis infection in neonatal pigs.

The results presented in Chapter V suggest that normal peristalsis may be important in the failure to establish infection with mucosalis in post-weaned pigs. The effects of pharmacologically induced hypomotility on the establishment of mucosalis in neonatal pigs was also investigated.

In the experiments described in this Chapter the infectivity of mucosalis for the neonatal pig, after oral dosing, was studied. The sites in which mucosalis could be demonstrated, and the numbers of mucosalis recovered, were investigated in relation to the interval post-dosing. The piglets were examined for evidence of PIA.

MATERIALS AND METHODS

Experimental Animals and Timing of Procedures

The litters of six sows were used in the experiments described in this chapter. Sows were from two sources; one sow (sow E) was from farm X, and the other five sows originated from farm A. The sows received water ad-lib, sow E was fed "PigMax Breeders' Cubes" (Scottish Agricultural Industries Ltd., Edinburgh) and the others "A.B.R.O. Breeders' meal" (Seafield Mill, Roslin). Sows were introduced into the isolation unit several days before farrowing. The six litters used in the experiments can be divided into three groups:

Group 1 - those which were orally dosed with mucosalis, before suckling their dams.

Group 2 - those which had suckled before oral dosing with mucosalis but which were dosed as soon as possible after birth.

Group 3 - those which had suckled before oral dosing with mucosalis, at three days of age. The piglets in group 3 also received benzetimide orally, at the time of dosing with mucosalis.

The number of piglets born to each sow, the treatment received by them and the timing is listed in Table 4a.

Table 4a Details of Piglets in Neonatal Infectivity Experiments.

Sow	Number of live piglets born	Treatment *	Time of Treatment	Deaths	Age when died or killed	Killed
A	15	5	-			
				72 hrs.		3, 6, 10 and 17 days
	10	C,M.	Pre-suckling	24 hrs, 24 hrs.	72 hrs	3, 6, 10, 13, 17 days
B	8	2	-			
				6 days		36 days
	6	M.	Pre-suckling			22, 22, 30, 30, 36, 44 days
C	13	3	-			
						43, 49, 56 days
	10	C,M.	Pre-suckling	48 hrs.		35, 35, 43, 49, 56, 64 days (4)
D	12	3	-			
				8 days		2, 54 days
	9	C,M.	Post-suckling (approx. 8 hrs.)			2, 32 days (2), 40 days (2) 48 days (2), 54 days (2)
E	4	1	-			
						2 days
	3	C,M	Post-suckling (approx. 24 hrs.)			4, 6, 8 days
J	9	C,M,B	Post-suckling (approx. 3 days)			36 days (2), 44 days (2) 51 days (2), 58 days (3)

* C = chalk M = mucosalis B = Benzetimide

Group 1. Litters dosed pre-suckling

This group includes the litters born to sows A, B and C. In all litters the undosed piglets were allowed to suckle naturally, the piglets to be dosed being removed at birth. The piglets in the litter of sow A were dosed approximately four hours post-partum, and then returned to the sow 135 minutes after dosing. The piglets in the litter of sow B were dosed two hours after birth and returned to the sow ninety minutes later. Dosing of the piglets in the litter of sow C was carried out approximately seventy five minutes after birth, and the piglets returned to the sow two hours later. From ten days of age the piglets were offered creep ("starter creep" - Seafield Mill, Roslin). The piglets in the litter of sow B were weaned at twenty two days of age, and those in the litter of sow C at twenty days old. After weaning the piglets were offered "A.B.R.O. Breeders' meal".

Mouth swabs were taken from a random selection of the piglets in the litters as follows:

Sow A at 5 days and 11 days old.

Sow B at 16 days of age

Sow C at 14 days and 27 days old.

A mouth swab was taken from sow A seven days before farrowing.

Group 2. Litters dosed post-suckling

This group includes the litters of sows D and E. The piglets in the litters of these two sows were allowed to suckle from birth. Those in the litter of sow D were dosed approximately eight hours post-partum. The litter of sow D was offered creep from ten

days of age, weaning was carried out at twenty days of age, and the piglets were fed "A.B.R.O. Breeders' meal" after weaning.

A mouth swab was taken from sow D six days before farrowing. Mouth swabs were taken from a random selection of the litter of sow D at 6 days, 12 days and 25 days of age.

Group 3. Litter dosed at three days old and which received benzetimide

The piglets in this group are from the litter of sow J. They were offered creep from twelve days of age, were weaned at twenty five days and were fed "A.B.R.O. Breeders' meal" after weaning. The piglets were usually weighed weekly, from seventeen days of age, and the cumulative sum technique, described in Chapter III, Section C, applied to the figures.

If G = gain in weight for one week

$$x_1 = G - 2.0$$

$$\text{and CUSUM} = X_1 + X_2 + X_3 \dots \text{etc.}$$

It was on the basis of these results that pigs were selected for killing, at the intervals depicted in Table 4a. The pigs killed at thirty six days of age were selected because their CUSUM values were about average for the litter. From that time the piglets with the worst CUSUM values were usually chosen.

Infecting Organism

The litters of sows A, B, C, D, and J all received cultures of mucosalis, strain 106/75 Sl. 10^{-3} grown in diphasic media. The litter of sow E was dosed with mucosalis strain 604/74 Ll -3. Estimates of the numbers of mucosalis used to dose the piglets are listed

below in Table 4b.

Table 4b The Numbers of Mucosalis used for Oral Dosing

Litters of:	Numbers of viable mucosalis administered to each piglet
Sow A	2.65×10^{10}
Sow B	1.50×10^{10}
Sow C	8.03×10^9
Sow D	6.50×10^9
Sow E	1.30×10^{10}
Sow J	8.25×10^9

Necropsy Procedure

This was carried out as described in Chapter II.

Sites Examined

On the piglets from the litter of sow E, bacteriological examination was carried out on the mucosa and contents of the M.S.I., T.S.I. and L.I. The mucosa only, at the sites M.S.I., T.S.I. and L.I. was examined bacteriologically from the piglets in the litters of sows A, B, C, D and J (except for the four piglets in the litter of sow C killed at 64 days of age, in which only the mouths were examined bacteriologically). Tissues from all three sites in these piglets were fixed for histological, immunofluorescent and electron microscopic examination. Before euthanasia, mouth swabs for bacteriological examination were taken from the piglets in the litters of sows B, C, D and J.

Bacteriological Examination

For the piglets of sow E, mucosal samples from the M.S.I., T.S.I. and L.I. were spread on N.B.G. plates at dilutions of $\frac{1}{20}$, $(\frac{1}{20})^2$ and $(\frac{1}{20})^3$ and on C.B.A. plates at $(\frac{1}{20})^2$, $(\frac{1}{20})^3$ and $(\frac{1}{20})^4$ dilutions. The contents from all three sites were spread on to C.B.A. plates at the $(\frac{1}{10})^4$ and $(\frac{1}{10})^5$ dilutions, and onto N.B.G. plates at the $\frac{1}{10}$, $(\frac{1}{10})^2$ and $(\frac{1}{10})^3$ dilutions.

The samples of mucosa from the M.S.I., T.S.I., and L.I. of the piglets of sow J, were plated out on to N.B.G. at the $\frac{1}{20}$, $(\frac{1}{20})^2$ and $(\frac{1}{20})^3$ dilutions, and on to C.B.A. or B.A. at the $\frac{1}{20}$, $(\frac{1}{20})^2$, $(\frac{1}{20})^3$ and $(\frac{1}{20})^4$ dilutions.

Filtration of mucosal samples from the M.S.I., T.S.I. and L.I. from the piglets in the litters of sows A, B, C and D was carried out. These filtered samples were used to inoculate B.A. and N.B.G. plates with 0.1 ml of inoculum. The homogenised, unfiltered mucosal samples were used to inoculate N.B.G. plates at the $\frac{1}{20}$, $(\frac{1}{20})^2$ and $(\frac{1}{20})^3$ dilutions.

RESULTS

Clinical Findings/Daily Observations

1. Litters dosed pre-suckling

No clinical signs were noted in piglets from the litters of sows A, B and C. A number of piglets were found dead (see Table 4a). The undosed piglet in the litter of sow B found dead at six days of age, was trapped between bars in the farrowing crate. The remaining piglets in these litters remained healthy and showed no evidence of enteric upset. They ate creep from an early age, and grew well throughout the duration of the experiment.

2. Litters dosed post-suckling

One undosed piglet in the litter of sow D was found dead at eight days of age, having previously shown no signs of illness. The remaining piglets of that litter, and of the litter of sow E all remained healthy throughout the experiments.

3. Litter dosed at three days old and which received benzetimide

From ten to fourteen days after dosing it became increasingly obvious that one of the piglets (272/76), in the litter of sow J was smaller compared to its litter mates. All the piglets in the litter were, however, healthy and showed no signs of disease. Later, between fourteen to twenty eight days post-dosing, it was noticed that some of the piglets were lean and that there was a distinct size variation within the group. Appetites were good throughout the experiment and there was no evidence of diarrhoea in any of the pigs. The pigs were weighed weekly from seventeen days of age, and the CUSUM technique applied to the figures. The weights and CUSUM values are presented in Table 4c.

Bacteriological Results

1. Litters dosed pre-suckling

Sow A. The results of bacteriological examination of the piglets in the litter of sow A are summarised in Table 4d. The estimated numbers of mucosalis recovered are presented in Table 4i. Mouth swabs were examined from four of the piglets five days after dosing, and from three of the piglets eleven days after dosing. No isolations of mucosalis, or other campylobacters, were made from these

Table 4c Body Weights and CUSUM Values for Pigs of the Litter of Sow J

Pig Reference	Days Post-Dosing											
	14		22		29		40*		47		54	
	W	C	W	C	W	C	W	C	W	C	W	C
234/76	11		13	0	15	0						
235/76	12		15	+1	16	0						
247/76	14.5		17	+0.5	17	-1.5	19	-2.5				
248/76	11		12	-1	12	-3	15	-3				
261/76	12		15	+1	14	-2	18	-1	19	-2		
262/76	14		16	0	16	-2	20	-1	21	-2		
270/76	12.5		14	-0.5	15.5	-1	19	-0.5	21	-0.5	25	+1.5
271/76	14		17	+1	18	0	20.5	-0.5	23	0	24	-1
272/76	5.5		8	+0.5	9	-0.5	11	-1.5	12	-2.5	16	-0.5

W = Body weight in lbs.

C = CUSUM

* Since 11 days between weighings, CUSUM = weight change -3.0.

Table 4d Litter ex. Sow A - Bacteriological Results

Pig Reference	Examined at Post-Dosing	Experimental Status *	Recovery of <u>Mucosalis</u> at Necropsy		
44/75 ⁺	< 24 hrs	D	-		
45/75 ⁺	< 24 hrs	D	+ L.I. contents		
46/75 ⁺	3 days	D	-		
47/75 ⁺	3 3 days	N.D.	No bacteriology carried out		
			M.S.I. Mucosa	T.S.I. Mucosa	L.I. Mucosa
48/75	3 days	N.D.	-	-	-
49/75	3 days	D	-	-	-
52/75	6 days	N.D.	-	-	-
53/75	6 days	D	-	-	+
54/75	6 days	D	-	-	-
59/75	10 days	N.D.	-	-	-
60/75	10 days	D	-	-	+
65/75	13 days	D	-	-	-
66/75	13 days	D	-	-	+
69/75	17 days	N.D.	-	+	-
70/75	17 days	D	-	-	-

* D = Dosed orally with mucosalisN.D. = Not dosed with mucosalis

+ All pigs were killed except for 44/75, 45/75, 46/75 and 47/75.

mouth swabs. Mucosalis was not isolated from a mouth swab from the sow, taken seven days before farrowing. Routine bacteriological examination did not reveal a cause for the death of piglets 44/75 and 46/75. Mucosalis was isolated in large numbers from the L.I. contents of pig 45/75, from which no other accepted bacterial pathogens were cultured.

Sow B. The results of bacteriological examination of the piglets in the litter of sow B are summarised in Table 4e. The estimated numbers of mucosalis recovered are presented in Table 4i. Mouth swabs examined from three of the dosed piglets, at sixteen days post-dosing, were positive for mucosalis, but a mouth swab from the control piglet examined at the same time was negative for mucosalis.

Sow C. The results of bacteriological examination of the piglets in the litter of sow C are summarised in Table 4f. Mouth swabs from piglets in this litter were taken at fourteen, twenty seven and sixty four days post-dosing, and the results are presented in Table 4g. The piglet found dead forty eight hours after dosing was considered, at post-mortem examination, to have been crushed and no bacteriological examination was undertaken.

The bacteriological results for the litters of piglets dosed before suckling are brought together in Table 4h. Mucosalis was isolated from the alimentary tract of five piglets which had been orally dosed, and from one piglet which had not been dosed. Mucosalis was isolated at post mortem from eight sites in seven piglets, as follows:

Table 4e Litter ex Sow B - Bacteriological Results

Pig Reference	Examined at Post-dosing	Experimental Status *	Recovery of <u>Mucosalis</u> at Necropsy			
			Mouth	M.S.I.	T.S.I.	L.I.
127/75	22 days	D	- ¹	-	-	-
128/75	22 days	D	-	-	-	-
143/75	30 days	D	+	-	-	-
144/75	30 days	D	+	-	-	+
148/75	36 days	D	-	-	-	-
149/75	36 days	N.D.	-	-	-	-
161/75	44 days	D	-	-	-	-

* D = Dosed orally with mucosalis
 N.D.= Not dosed with mucosalis

¹ Mucosalis not recovered, but other catalase negative, slide agglutination negative campylobacters were isolated.

Table 4f Litter ex Sow C - Bacteriological Results

Pig Reference	Examined at Post-dosing	Experimental Status *	Recovery of <u>Mucosalis</u> at Necropsy			
			Mouth	M.S.I.	T.S.I.	L.I.
159/75	35 days	D	-	-	-	-
151/75	35 days	D	-	-	-	-
163/75	43 days	N.D.	-	-	-	-
164/75	43 days	D	-	-	-	-
168/75	49 days	D	-	-	-	-
169/75	49 days	N.D.	- ¹	-	-	-
173/75	56 days	N.D.	-	-	-	-
174/75	56 days	D	-	-	-	-

* D = Dosed orally with mucosalis

N.D.= Not dosed with mucosalis

¹ Mucosalis was not recovered from the mouth, but other catalase negative, slide agglutination negative campylobacters were isolated. A mouth swab from this pig had yielded mucosalis at 27 days post-dosing.

The dosed piglet found dead at 48 hours post-dosing, and the four dosed piglets killed at 64 days post-dosing are not included in this table.

Table 4g Litter ex Sow C - Bacteriological Results

Interval Post-dosing	Experimental Status *	Recovery of <u>Mucosalis</u> from Mouth Swabs
14 days	1 x N.D.	- for <u>mucosalis</u> , but other catalase negative, slide agglutination negative campylobacters isolated.
	3 x D	2/3 + <u>mucosalis</u>
27 days	1 x N.D.	+ <u>mucosalis</u>
	3 x D	1/3 + <u>mucosalis</u>
64 days	4 x D	4/4 - for <u>mucosalis</u>
		2/4 had catalase negative, slide agglutination negative campylo- bacters in the mouth. One of these two pigs had been + for <u>mucosalis</u> at 27 days.

* D = Dosed orally with mucosalis

N.D. = Not dosed with mucosalis

Table 4h Pre-suckled Piglets ex Sows A, B and C

Sow	Numbers of Pigs Examined		Numbers Dying		Numbers from Whose Alimentary Tract <u>Mucosalis</u> was Isolated	
	D *	N.D.*	D	N.D.	D	N.D.
A	10	5	3	1	4/9	1/4
B	6	2	0	1	1/6	0/1
C	10	3	1	0	0/5	0/3
Totals	26	10	4	2	5/20	1/8

* D = Dosed orally with mucosalis

N.D. = Not dosed with mucosalis

L.I.	5	(mucosa 4, contents 1)
T.S.I.	1	
Mouth	2	(also recovered from the large intestine of one of these).

The piglet in which mucosalis was isolated from the T.S.I. had not been orally dosed. Pigs were killed at intervals up to fifty six days, and bacteriological examination carried out on the alimentary tract, but mucosalis was only isolated up to thirty days post-dosing. The numbers of mucosalis isolated from the alimentary mucosa of those pigs, which were positive for mucosalis, are presented in Table 4i. Also recorded there are the samples and the media on which the isolations were made.

Twenty three mouth swabs were taken from piglets in the three litters, and mucosalis was isolated from seven of these, as shown in Table 4j (not including those taken at euthanasia of the pigs). Mucosalis was isolated from the mouth up to thirty days post-dosing.

The numbers of colonies of mucosalis on the plates were often small, and on B.A. plates could have been overgrown by other organisms. Overgrowth by other members of the enteric flora was less of a problem with the N.B.G. plates, but again the numbers of colonies of mucosalis recovered were usually small.

In conclusion it can be seen that in piglets dosed orally with mucosalis before suckling, mucosalis can be recovered in low numbers from the alimentary tract mucosa, especially of the L.I., of some of these piglets. It may also be isolated from the intestinal mucosa of piglets which were not orally dosed with mucosalis, but which were kept with their orally dosed litter mates. Isolations were made from

Table 4i The Numbers of Mucosalis Recovered, and the Samples from which Isolations were Made, for Those Piglets in Litters Dosed Pre-suckling.

Pig Reference	Site	Interval Post-dosing	Sample/Dilution and Media	Numbers of Viable <u>Mucosalis</u> per Gram of Mucosa
53/75	L.I. Mucosa	6 days	Unfiltered mucosa $(\frac{1}{20})^2$ N.B.G.	$\geq 3.4 \times 10^4$
60/75	L.I. Mucosa	10 days	Unfiltered mucosa $\frac{1}{20}$ and $(\frac{1}{20})^2$ N.B.G.	$\geq 1.92 \times 10^4$
66/75	L.I. Mucosa	13 days	Unfiltered mucosa $\frac{1}{20}$ N.B.G.	$\geq 4 \times 10^2$
69/75	T.S.I. Mucosa	17 days	Unfiltered mucosa $\frac{1}{20}$ N.B.G.	$\geq 2 \times 10^2$
144/75	L.I. Mucosa	30 days	Filtered mucosa $\frac{1}{10}$ N.B.G.	$\geq 1 \times 10^2$

Table 4j Recovery of Mucosalis from Mouth Swabs from those Litters Dosed Pre-suckling.

Litter of Sow	Number of Mouth Swabs Taken	Number from which <u>Mucosalis</u> Isolated	<u>Mucosalis</u> Isolations Made On	
			B.A.	N.B.G.
A	7	0	0	0
B	4	3	1	2
C	12	4	0	4
Totals	23	7	1	6

Mucosalis was also isolated from the mouths of two piglets (143/75 and 144/75) of sow B at necropsy. One of these recoveries was made on B.A. and the other on N.B.G.

the intestinal mucosa, up to thirty days after oral dosing. Mucosalis can also be isolated from the mouths of these piglets, both those which were orally dosed and their in contact, but undosed litter mates. In these experiments mucosalis was isolated from the oral cavity up to thirty days post-dosing.

The usefulness of the inhibitory media is stressed; all five recoveries from the intestinal mucosa were made on N.B.G. plates. Four of these isolates were from unfiltered mucosal samples, the fifth being from a filtered mucosal sample. Mucosalis was not isolated on non-inhibitory media inoculated from the same samples, although this was not done on every occasion. On the nine occasions that mucosalis was isolated from the oral cavity seven of these recoveries were made on N.B.G. and on the other two occasions on B.A. (see Table 4j). For both the intestinal mucosa and the mouth on no occasion in these experiments was mucosalis isolated on both the inhibitory and non-inhibitory media inoculated from the same sample. On B.A. or C.B.A. plates (non-inhibitory media) inoculated with the lower dilutions of mucosa there was often a heavy growth, in which small numbers of mucosalis could easily have been overgrown and difficult to recognise. This was not usually a problem with the N.B.G. plates (inhibitory media) inoculated with the same low dilutions of mucosa, and recovery and recognition of small numbers of mucosalis was easier. The use of N.B.G. plates proved to be most useful in the recovery of mucosalis, when only small numbers were present in the sample.

2. Litters dosed post-suckling

Sow D. The results of the bacteriological examination of the litter of sow D are summarised in Table 4k. Mucosalis was not recovered from the control piglet, found dead at eight days of age, and no other accepted bacterial pathogens were isolated. In the dosed piglet killed at forty days of age (87/75) mucosalis was isolated from the mucosa of the M.S.I. Also isolated from this same sample were other campylobacters, which were catalase negative in slide and slope catalase tests, but which did not agglutinate in slide and tube agglutination tests with standard mucosalis antiserum (1248/72 2C2). These isolates did, however, agglutinate with antiserum prepared against mucosalis strain 982/76 (see Chapter III, Section B) in both slide and tube agglutination tests. Mouth swabs were taken from some members of the litter of sow D at six days, twelve days and twenty five days post oral dosing with mucosalis, and also from the sow six days before farrowing. The results of bacteriological examination of these are summarised in Table 4l.

Sow E. The bacteriological results of examination of piglets from sow E are summarised in Table 4m.

The results of the bacteriological examinations and the recovery of mucosalis from the piglets of sows D and E are summarised in Table 4n. Mucosalis was isolated from the alimentary tract of four of the piglets which had been orally dosed, and from two which had not been orally dosed, with mucosalis. It is stressed that these latter, undosed piglets, were maintained with their dosed litter mates. Mucosalis was isolated at post mortem examination from nine sites in

Table 4k Litter ex Sow D - Bacteriological Results

Pig Reference	Examined at Post-dosing	Experimental Status *	Recovery of <u>Mucosalis</u> at Necropsy			
			Mouth	M.S.I.	T.S.I.	L.I.
42/75	2 days	N.D.	-	-	+	-
43/75	2 days	D	-	+	+	-
79/75	32 days	D	-	-	-	-
80/75	32 days	D	-	-	-	+
87/75	40 days	D	-	+ ¹	-	-
88/75	40 days	D	+	-	-	-
97/75	48 days	D	-	-	-	-
98/75	48 days	D	-	-	-	-
102/75	54 days	N.D.	-	-	-	-
103/75	54 days	D	-	-	-	-
104/75	54 days	D	-	-	-	-

* D = Dosed orally with mucosalis
 N.D. = Not dosed with mucosalis

¹ In addition to serologically typical mucosalis, other catalase negative campylobacters were recovered, which were agglutinated by 982/76 mucosalis antiserum.

The control piglet (57/75) which died at eight days of age is not included in this table.

Table 41 Litter ex Sow D - Bacteriological Results

Time of Sampling	Animals	Recovery of <u>Mucosalis</u> from Mouth Swabs
6 days before farrowing	Sow	<u>Mucosalis</u> not recovered, but other catalase negative, slide agglutination negative campylobacters present
6 days post-dosing	1 x N.D.* 3 x D *	4/4 <u>mucosalis</u> not recovered. 2/4 other catalase negative, slide agglutination negative campylobacters present
12 days post-dosing	1 x N.D. 2 x D	1/1 + <u>mucosalis</u> 1/2 + <u>mucosalis</u>
25 days post-dosing	4 x D	3/4 + <u>mucosalis</u>

* D = Dosed orally with mucosalis

N.D. = Not dosed with mucosalis

Table 4m Litter ex Sow E - Bacteriological Results

Pig Reference	Examined at Post-dosing	Experimental Status *	Recovery of <u>Mucosalis</u> at Necropsy			
			M.S.I. Contents	Mucosa Contents	T.S.I. Mucosa Contents	L.I. Mucosa Contents
195/74	24 hours	N.D.	+	-	-	-
198/74	3 days	D	-	-	-	-
200/74	5 days	D	-	+	+	-
201/74	7 days	D	-	-	-	-

* D = Dosed orally with mucosalis
N.D. = Not dosed with mucosalis

Table 4n Post-suckled Piglets ex Sows D and E

Sow	Numbers of Pigs Examined		Numbers Dying		Numbers from Whose Alimentary Tract <u>Mucosalis</u> was Isolated	
	D *	N.D. *	D	N.D.	D	N.D.
D	10	2	0	1	3/10	1/2
E	3	1	0	0	1/3	1/1
Totals	13	3	0	1	4/13	2/3

* D = Dosed orally with mucosalis
 N.D. = Not dosed with mucosalis

seven piglets as follows:

M.S.I. mucosa	-3
M.S.I. contents	-1
T.S.I. mucosa	-3
L.I. mucosa	-1
Mouth	-1

In two pigs mucosalis was isolated from both the M.S.I. mucosa and the T.S.I. mucosa, at euthanasia.

Piglets were killed, and the alimentary tracts subjected to bacteriological examination up to fifty four days post oral dosing with mucosalis. Mucosalis was recovered from the intestinal mucosa up to forty days post-dosing. It was also recovered from the alimentary tract (M.S.I. contents) of an undosed piglet, as early as twenty four hours after dosing of this piglet's littermates. The numbers of mucosalis recovered from the alimentary tract mucosa of those piglets, which were culturally positive for mucosalis, is presented in Table 4p. Also recorded there are the samples from which the recoveries, and the media on which the isolations were made. Eleven mouth swabs were taken from piglets in the litter of sow D, and mucosalis was isolated from five of these (This does not include mouth swabs taken at euthanasia of the pigs). Mucosalis was recovered from the oral cavity of piglets in the litter of sow D, up to forty days post oral dosing.

In conclusion, it can be seen that in piglets dosed orally with mucosalis in the immediate post-partum period, but after suckling the sow, mucosalis can be recovered in low numbers from the alimentary

Table 4p The Numbers of Mucosalis Recovered, and the Samples from which Isolations Were Made for Those Piglets in Litters Dosed Post-suckling

Pig Reference	Site	Interval Post-dosing	Sample/Dilution and Media	Numbers of Viable <u>Mucosalis</u> per Gram of Mucosa (or Contents)
42/75	T.S.I. Mucosa	2 days	Filtered 1/10 Mucosa	$\geq 1 \times 10^2$
43/75	M.S.I. Mucosa	2 days	Unfiltered 1/20 Mucosa	$\geq 3.2 \times 10^3$
	T.S.I. Mucosa		Filtered 1/10 Mucosa	$\geq 5 \times 10^2$
80/75	L.I. Mucosa	32 days	Unfiltered 1/20 Mucosa	$\geq 2 \times 10^2$
87/75	M.S.I. Mucosa	40 days	Unfiltered 1/20 Mucosa	$\geq 2 \times 10^2$ and also $\geq 6 \times 10^2$ of <u>mucosalis</u> type 982/76
195/74	M.S.I. Contents	1 day	Unfiltered (1/10) ³ Contents	$\geq 3.5 \times 10^4$
200/74	M.S.I. Mucosa	5 days	Unfiltered 1/20 Mucosa	$\geq 2 \times 10^2$
	T.S.I. Mucosa		Unfiltered (1/20) ² Mucosa	$\geq 3.6 \times 10^4$

tract mucosa of some of these piglets up to forty days post dosing. There was also rapid spread to undosed litter mates, so that mucosalis was isolated from the M.S.I. contents of one of these undosed piglets at twenty four hours after its litter mates were exposed, and from the T.S.I. mucosa of another undosed piglet at forty eight hours after oral dosing of its litter mates. Similarly, isolations of mucosalis were made from the mouths of both orally dosed and undosed piglets. Mucosalis was recovered from the oral cavity up to forty days post dosing, in piglets of the litter of sow D.

As in the pre-suckled experiments, the usefulness of the inhibitory media (N.B.G.) was seen; all intestinal recoveries of mucosalis were made on this media. For the recoveries from the mouth, the five isolations detailed in Table 4l, were made on N.B.G., but the recovery from the mouth of pig 88/75, at euthanasia, was made on B.A. Five of the seven mucosalis recoveries from the intestine, were achieved from unfiltered mucosal samples.

3. Litter dosed at three days old and which received benzetimide

Sow J. The results of the bacteriological examination of the litter of sow J are summarised in Table 4q. Mucosalis was only recovered from one piglet (261/76), from the L.I. mucosa. Recovery was made from an unfiltered mucosal sample at both the $\frac{1}{20}$ and $(\frac{1}{20})^2$ dilutions, on N.B.G. The numbers of mucosalis isolated were $\geq 4.0 \times 10^3$ viable mucosalis per gram of mucosa. Mucosalis was recovered from the L.I. mucosa of pig 261/76 forty eight days after oral dosing with mucosalis. Mouth swabs were taken from each of the nine piglets at euthanasia, but mucosalis was not isolated.

Table 4a Litter ex Sow J - Bacteriological Results

Pig Reference	Examined at Post-dosing	Recovery of <u>Mucosalis</u> at Necropsy			
		Mouth	M.S.I.	T.B.I.	L.I.
234/76	33 days	-	-	-	-
235/76	33 days	-	-	-	-
247/76	41 days	- ¹	-	-	-
248/76	41 days	- ¹	-	-	-
261/76	48 days	-	-	-	+
262/76	48 days	-	-	-	-
270/76	55 days	-	-	-	-
271/76	55 days	-	-	-	-
272/76	55 days	-	-	-	-

¹ Mucosalis was not recovered from the mouth swab, but other catalase negative, slide agglutination negative campylobacters were isolated.

The piglet found dead at three days of age (233/76) is not included in this table, since no bacteriological examination was carried out.

Pathological Results

Gross Findings

The post mortem findings in those piglets which were found dead in the course of the experiments are presented in Table 4r. This includes piglets from both the pre-suckled and post-suckled studies, none of the piglets in the litter dosed at three days of age died after dosing. The gross findings in the sacrificed animals of all three groups of piglets will be considered together. There were no definite adenomatous changes seen in any of the pigs killed. With increasing age the Peyer's patches, especially of the terminal ileum, became very prominent and caused thickening of the surface, which appeared superficially like adenomatous mucosal proliferation. With experience it became possible to differentiate the two types of thickening, on the basis that if the thickening was due to adenomatous mucosal proliferation, then it could not be effaced by stretching of the wall, in contrast, thickening due to lymphoid hyperplasia was obliterated by stretching of the wall. The thickening due to adenomatous mucosal proliferation also had a more solidly cellular appearance, than that due to lymphoid hyperplasia. On close examination lymphoid follicles were seen on the surface and cut surface of Peyer's patches. Similar accumulations of lymphoid tissue appeared in the caecum, at the ileo-caeco-colic valve. In some of the younger piglets clumping of the villi occurred and gave the impression that the mucosa was thickened. However, if a portion of the intestine was suspended in buffered saline and examined under a low-power dissecting microscope, this clumping of villi could easily be appreciated.

Table 4r Gross Pathological Findings in Those Piglets Found Dead in the Neonatal Piglet Infectivity Experiments

Sow	Pig Reference	Experimental Status *	Examined at Post-dosing	Necropsy Findings	Mucosalis Recovered
A	44/75	D	1 day	No significant findings	-
	45/75	D	1 day	Cyanotic. The L.I. was grossly dilated and distended with fluid and gas. No milk in the alimentary tract - not suckled.	+ L.I. contents
	46/75	D	3 days	No significant findings	-
	47/75	N.D.	3 days	Evidence of crushing	Not done
B	-	N.D.	6 days	No necropsy. This piglet became trapped, and the head swollen leading to asphyxiation.	Not done
	100/75	D	2 days	Evidence of crushing. Severe superficial bruising. Rupture of liver and kidney	Not done
D	57/75	N.D.	8 days	No significant findings	-

* D = Dosed orally with mucosalis
 N.D. = Not dosed with mucosalis.

As the pigs aged the lymph node chain in the mesentry, especially those draining the terminal ileum in the region of the ileo-caeco-colic junction became larger and cellular, with a follicular pattern easily discernible on the surface and cut-surface.

Only one exception to the absence of mucosal thickening was observed. In pig 248/76 (litter of sow J), the mucosa of the large intestine was thought to be slightly thickened.

Histopathology

1. Litters dosed pre-suckling

There were no definite adenomatous changes in the sections of alimentary tract examined from any of the piglets in the litters of sows A, B and C. The histological appearance of the alimentary tract in these piglets conformed to that documented in the literature. In the small intestine of the younger pigs there were long slender villi and relatively short crypts, with the lamina propria containing few cells. In these pigs there was vacuolation of the intestinal epithelium, especially of the terminal ileum. This vacuolation has been well described in the upper small intestine (Staley, Jones and Marshall, 1968; Staley, Jones and Corley, 1969a; Clarke and Hardy, 1971) and also in the terminal small intestine (Hardy, Hockaday and Tapp, 1971; Clarke and Hardy, 1971; Moon, 1972; Moon, Kohler and Whipp, 1973), where it persists for a longer time. As the pigs aged the number of cells in the lamina propria increased, but varied considerably. These cells included eosinophils (occasionally in large numbers), plasma cells, lymphocytes, macrophages and occasional

neutrophils. After weaning the villi became much shorter and the crypts elongated, with the crypt epithelium showing an increased mitotic activity. The lamina propria at that time also showed a marked increase in cellularity. A small number of glands contained debris and degenerating neutrophils. These findings are consistent with the descriptions of the histology of the small intestine in the post-weaned pig by Kenworthy (1971 and 1976).

The large intestine also conformed to descriptions of the histology of this area (Donnellan, 1965) and similar changes to those described in the small intestine, were seen after weaning. There was a marked increase in the cellularity of the lamina propria, and the crypts were lengthened with an increase in the mitotic activity of the epithelium. The changes in the large intestines of a number of pigs differed and will be described below.

In those pigs killed up to two to three weeks of age, drainage lymph nodes of the alimentary tract, especially those serving the terminal ileum, showed moderate to large numbers of neutrophil polymorphs present within the sinuses. This was not a feature of the older pigs, but eosinophils in varying numbers were often present in the lymph node sinuses of these animals.

Figs 144/75, 148/75 and 149/75 (Sow B)

In the large intestines of these pigs there were foci of surface epithelial damage, together with degenerative changes involving the deeper crypt epithelium in the same areas. There were increased numbers of neutrophil polymorphs, plasma cells, lymphocytes and macrophages throughout the lamina propria, with the first named

especially prominent, in areas of epithelial damage. Occasionally the gland lumen contained cell debris and degenerating neutrophils, and migrant neutrophils were present within the epithelium. Eosinophils were also seen within the epithelium, especially in those glands containing cell debris. Some of the glands had a crowded appearance, but were certainly not genuinely adenomatous. Other areas appeared more normal, with a substantial mucus-secreting cell population within the epithelium. The epithelium, deep into the base of the crypts, was made up of goblet cells in some glands.

Pigs 164/75, 168/75, 173/75 and 174/75 (Sow C)

In the large intestines of these pigs there was also epithelial damage, and associated with this there was an infiltration of neutrophil polymorphs. They differed from the last described group in that in some areas the surface epithelium was flattened and cuboidal rather than columnar. The lamina propria was mostly densely cellular, with an increase in plasma cells, lymphocytes and macrophages. In other areas the lamina propria was oedematous. There were areas of localised infiltration of cells into the sub-mucosa, predominantly lymphocytes and plasma cells. Occasional glands contained cell debris and degenerating neutrophils.

2. Litters dosed post-suckling

In the younger piglets of the litters of sows D and E, vacuolation of the intestinal epithelium was seen identical to that seen in the piglets dosed before suckling. Also seen within epithelial cells were inclusions, some spherical and others elongate or

spindle-shaped, which appeared to be the same as structures described in the literature as: lamellar bodies (Clarke and Hardy, 1971), inclusion bodies and spicules (Hardy, Hockaday and Tapp, 1971) and crystals (Staley, Jones and Corley, 1969c). No attempt was made to further examine or identify these bodies.

The histological appearance of the intestines of these piglets resembled that described for the piglets dosed pre-suckling. In a number of piglets the changes in the large intestine were more marked and these are considered below.

Figs 80/75, 88/75, 93/75 and 102/75 (Sow D)

There was a marked increase in the cells in the lamina propria of these pigs, especially of plasma cells. There was some damage to the surface epithelium, and in these areas neutrophil polymorphs were seen in the lamina propria. Occasional scattered glands had cell debris and degenerating neutrophils in the lumen.

Fig 201/74 (Sow E)

In some areas of the large intestine the surface epithelium was flattened and more cuboidal than columnar in appearance. In association with these changes there was an increased number of neutrophil polymorphs present within the lamina propria.

3. Litter dosed at three days old and which received benzetimide

The piglets of the litter of sow J were all killed after weaning, and the mucosal architecture of the intestine was essentially the same as that of the pigs in groups 1 and 2, killed at a corresponding age.

This following section contains a more detailed description of the histological features of the intestines which differed from those already described.

Pigs killed at 36 days

Pigs 234/76 and 235/76

In the large intestine there was a variation in gland size and shape, and although some glands had a crowded appearance no definitely adenomatous glands were seen. There were neutrophil polymorphs scattered throughout the lamina propria, and some glands contained cell debris and degenerating neutrophils.

Pigs killed at 44 days

Pig 247/76 M.S.I. and T.S.I.

In these sites there was severe villous atrophy, and a number of glands contained cell debris and degenerating neutrophils. In the lamina propria surrounding these glands there was an increased number of neutrophils and eosinophils and these cells were also seen within the epithelium of some of these glands.

L.I.

There were many mitotic figures within the epithelium, and some glands had a crowded appearance, but were not definitely adenomatous. However, there was a substantial goblet cell population present within these glands. Foci of epithelial damage, with neutrophil infiltration were also seen.

Fig 248/76 M.S.I. and T.S.I.

In sections of both M.S.I. and T.S.I., but especially of the former, occasional adenomatous glands were seen. These were easily recognised from the surrounding glands, since they were larger, had an increased number of cells and mostly lacked goblet cells. In some of these adenomatous glands there were occasional mucus-secreting cells present, and often there was cell debris and neutrophils present in the lumen. Neutrophils and eosinophils were also seen within the epithelium of these glands. In silver-stained sections curved bacterial forms, resembling vibrios were seen in the apical cytoplasm of the epithelial cells of the adenomatous glands. Also occasional vibrios were seen in the inflammatory cells in the gland lumen.

L.I.

There was some variation in gland size and shape, and many of the glands had a crowded appearance, but there were always mucus-secreting cells present. Occasional crypts contained cell debris and degenerating neutrophils. There was a very dense cellular infiltration into the lamina propria of lymphocytes, plasma cells and macrophages. This latter probably accounted for the thickening of the mucosa observed grossly.

Pigs killed at 51 days

Fig 261/76

There was severe villous atrophy and crypt hyperplasia in the small intestine (M.S.I. and T.S.I.), with some glands containing cell debris. In the large intestine gland size and shape varied, and some of the glands had a crowded appearance, but were not definitely

adenomatous. In occasional crypts in the large intestine, there was cell debris and neutrophils.

Pig 262/76

In sections examined from the M.S.I. and T.S.I. one adenomatous gland was found in each site. In both cases the gland contained cell debris and degenerating neutrophils, and neutrophils within the epithelium. The surrounding crypts were markedly hyperplastic and some contained cell debris. The villi showed severe atrophy. Curved bacteria of vibrio morphology were seen in the apical cytoplasm of the epithelial cells of adenomatous glands, in silver stained sections. These were absent from surrounding normal glands.

In the large intestine no adenomatous glands were seen, but there was an increase in the cellularity of the lamina propria, some crypts contained cell debris and associated with foci of surface damage, there were neutrophils present.

Pigs killed at 58 days

Pigs 270/76, 271/76 and 272/76

The histological appearance of all sites (M.S.I., T.S.I. and L.I.) examined in these pigs conformed basically to the description for pig 247/76.

Electron Microscopy

Tissues from piglets of the litters of sows A, B, D and E were examined. Only a limited amount of tissue was examined, and those cases chosen were those in which mucosalis had been recovered

bacteriologically. Not all cases from which mucosalis had been isolated were examined by electron microscopy. No intracellular bacteria were seen. The ultrastructure of the intestinal epithelium was in accordance with the descriptions in the literature. In the samples from pigs killed at an early age the epithelium had a well-developed system of apical tubules, vesicles and vacuoles (Kraehenbuhl and Campiche, 1969), which persisted longer in the ileum (Staley, 1969; Moon, Kohler and Whipp, 1973). The ultrastructure of the upper small intestinal epithelium of neonatal pigs (Staley, Jones and Marshall, 1968; Staley, Jones and Corley, 1969a) and of the ileum (Hardy, Hockaday and Tapp, 1971; Moon, 1972), has been described, and the cases examined here conformed to this. Similarly, the ultrastructure of the small intestine of the older pigs, both before and after weaning, resembled published accounts (Kenworthy, Stubbs and Syme, 1967). The large intestine was essentially the same as the descriptions of human tissue (Donnellan, 1965; Toner, Carr and Wyburn, 1971).

Tissues from piglets in the litters of sows C and J were not examined.

Selected sites were chosen for examination, which had on immunofluorescent staining, shown positive fluorescence in the lamina propria. In these sites no bacteria were seen within macrophages in the lamina propria, or in the overlying epithelium.

Immunofluorescent Results

1. Litters dosed pre-suckling

There was no specific fluorescence seen in cryostat sections of intestine from the piglets of sow C which were examined. Non-specific fluorescence, which could be altered by changing the exciting filters as described in Chapter III, was observed.

In the piglets from the litters of sows A and B occasional very small areas of what appeared to be particulate fluorescence were seen. These only involved a few cells in any gland, and were less bright in comparison to the positive controls stained at the same time. However, brighter specific fluorescence was seen in a number of piglets from the two litters. This was located in the lamina propria (Figure 55), appeared to be intracellular and was particulate (Figure 56). It was as bright as the fluorescence in positive controls and was apparently arranged around a central nucleus. It was concluded that this was mucosalis antigen present within macrophages or other phagocytic cells in the lamina propria.

The immunofluorescent findings for the piglets in these three litters are summarised in Tables 4s, 4t and 4u. In these tables they are compared with the results of the other examinations. There is a reasonable correlation between the demonstration of mucosalis by immunofluorescence and the recovery of mucosalis, if one considers the pigs without reference to the particular site (see below). Any fluorescence in the epithelium was, however, less bright than in the positive controls, and only present in small amounts. In contrast to this, the fluorescence in the lamina propria was brighter and

Table 4s Litter ex Sow A - Summary of Results

Pig Reference	Examined at Post-dosing	Experimental Status	Bacteriology	Pathology/Histopathology	Immunofluorescence
45/75	< 24 hrs	D	+ L.I. contents	L.I. grossly dilated	Not done
48/75	3 days	N.D.	-	N.A.D.	-
49/75	3 days	D	-	N.A.D.	-
52/75	6 days	N.D.	-	N.A.D.	-
53/75	6 days	D	+ L.I. Mucosa	N.A.D.	+/- deeper glands L.I.
54/75	6 days	D	-	N.A.D.	-
59/75	10 days	N.D.	-	N.A.D.	+/- L.I.
60/75	10 days	D	+ L.I. Mucosa	N.A.D.	+/- M.S.I.
65/75	13 days	D	-	N.A.D.	+/- L.I.
66/75	13 days	D	+ L.I. Mucosa	N.A.D.	-
69/75	17 days	N.D.	+ T.S.I. Mucosa	N.A.D.	++/++ M.S.I. - Lamina Propria
70/75	17 days	N.D.	-	N.A.D.	++/++ M.S.I. - Lamina Propria
					+/- L.I.
					++/- occasional macrophages in the lamina propria.

Table 4t Litter ex Sow B - Summary of Results

Pig Reference	Examined at Post-dosing	Experimental Status	Bacteriology	Pathology/Histopathology	Immunofluorescence
127/75	22 days	D	-	N.A.D.	-
128/75	22 days	D	-	N.A.D.	-
143/75	30 days	D	+ Mouth	N.A.D.	L.I. ? +/- lumina of some glands
144/75	30 days	D	+ Mouth + L.I. Mucosa	L.I. - Some foci of surface epithelial damage, and + neutrophils.	++/- M.S.I. - Lamina Propria
148/75	36 days	D	-	L.I. - As 144/75	M.S.I., T.S.I. ++/- in the lamina propria L.I. +/- occasional crypts.
149/75	36 days	N.D.	-	L.I. - As 144/75	M.S.I. ++/- in the lamina propria
161/75	44 days	D	-	N.A.D.	-

Table 4u Litter ex Sow C - Summary of Results

Pig Reference	Examined at Post-dosing	Experimental Status	Bacteriology	Pathology/Histopathology	Immunofluorescence
150/75	35 days	D	-	N.A.D.	-
151/75	35 days	D	-	N.A.D.	-
163/75	43 days	N.D.	-	N.A.D.	-
164/75	43 days	D	-	L.I. Some surface epithelial damage, and + neutrophils	-
168/75	49 days	D	-	L.I. As 164/75	-
169/75	49 days	N.D.	-	N.A.D.	-
173/75	56 days	N.D.	-	L.I. As 164/75	-
174/75	56 days	D	-	L.I. As 164/75	-

occasionally present in considerable quantities. This fluorescence in the lamina propria was seen from as early as thirteen days post-dosing, up to thirty six days post-dosing.

2. Litters dosed post-suckling

The findings in these two litters (Tables 4v and 4w) were similar to those reported for the pre-suckled piglets. In no case was convincing fluorescence seen in the intestinal epithelium, but bright fluorescence was seen in the lamina propria at thirty two days and forty days post-dosing.

3. Litters dosed at three days old and which received benzetimide

The immunofluorescent findings are presented in Table 4x. In a number of these pigs there appeared to be positive specific fluorescence within some of the glands in the large intestine. This fluorescence was particulate and on occasions some appeared as if it might be intracellular in the epithelium. Other than for pig 248/76, convincing fluorescence was not seen in the intestinal epithelium. In pig 248/76 the adenomatous glands showed bright particulate fluorescence in the apical cytoplasm of the epithelial cells.

Correlation between the recovery of mucosalis and its demonstration by immunofluorescence

If one compares the results for all of these piglets without reference to particular sites, i.e., both those dosed before suckling, after suckling and those dosed at three days of age (Tables 4s to 4x),

Table 4v Litter ex Sow D - Summary of Results

Pig Reference	Examined at Post-dosing	Experimental Status	Bacteriology	Pathology/Histopathology	Immunofluorescence
42/75	2 days	N.D.	+ T.S.I. Mucosa	N.A.D.	M.S.I. +/- L.I. ? +/- lumen
43/75	2 days	D	+ M.S.I. Mucosa + T.S.I. Mucosa	N.A.D.	+/- M.S.I. and T.S.I. +/- L.I. in epithelium and lumen
79/75	32 days	D	-	N.A.D.	+/- M.S.I., in the lamina propria
80/75	32 days	D	+ L.I. Mucosa	L.I. - some surface epithelial damage and + neutrophils	+/- M.S.I. +/- L.I.
87/75	40 days	D	+ M.S.I. Mucosa	N.A.D.	-
88/75	40 days	D	+ Mouth	L.I. As 80/75	M.S.I. and T.S.I. +/- in the lamina propria
97/75	48 days	D	-	N.A.D.	-
98/75	48 days	D	-	L.I. As 80/75	-
102/75	54 days	N.D.	-	L.I. As 80/75	-
103/75	54 days	D	-	N.A.D.	-
104/75	54 days	D	-	N.A.D.	-

Table 4w Litter ex Sow E - Summary of Results

Pig Reference	Examined at Post-dosing	Experimental Status	Bacteriology	Pathology/Histopathology	Immunofluorescence
195/74	24 hrs	N.D.	+ M.S.I. contents	N.A.D.	? +/- T.S.I. +/- L.I.
198/74	3 days	D	-	N.A.D.	+/- M.S.I.
200/74	5 days	D	+ M.S.I. Mucosa + T.S.I. Mucosa	N.A.D.	-
201/74	7 days	D	-	Surface epithelium flattened, some areas, and + neutrophils	+/- M.S.I., T.S.I. and L.I.

Table 4x Litter ex Sow J - Summary of Results

Pig Reference	Examined at Post-dosing	Bacteriology	Pathology/Histopathology	Immunofluorescence
234/76	36 days	-	L.I. Some epithelial destruction and + neutrophils	M.S.I., T.S.I. occasional glands +/- L.I. lumen ++/--
235/76	36 days	-	L.I. Some epithelial damage and + neutrophils	M.S.I., T.S.I., occasional glands +/- L.I. lumen ++/-- particulate.
247/76	44 days	-	L.I. Some epithelial damage and + neutrophils. Glands have a crowded appearance	T.S.I. +/- some cells, some glands L.I. ++/-- lumen.
248/76	44 days	-	M.S.I. and T.S.I. - Occasional adenomatous glands. L.I. - Grossly slightly thickened histology as 247/76	T.S.I. - ++/++ in adenomatous glands. Other glands show a little fluorescence in some cells.
261/76	51 days	+ L.I. Mucosa	L.I. As 247/76	L.I. ++/-- lumina, some glands
262/76	51 days	-	M.S.I. and T.S.I. Adenomatous glands very occasionally seen. L.I. As 247/76	-
270/76	58 days	-	L.I. as 247/76	L.I. ++/-- some glands, mostly luminal
271/76	58 days	-	L.I. as 247/76	L.I. ++ $\frac{1}{2}$ -- lumina, some glands
272/76	58 days	-	L.I. as 247/76	-

for the recovery of mucosalis and its demonstration by immunofluorescence, then the correlation between the two parameters can be estimated. Since each of the variables is a dichotomy the phi coefficient was used (Downie and Health, 1974).

Comparison of Positive Immunofluorescence and recovery of Mucosalis from individual pigs:

	CULTURAL RECOVERY OF <u>MUCOSALIS</u>		
	+		-
	<hr/>		
DEMONSTRATION OF <u>MUCOSALIS</u> BY IMMUNOFLUORESCENCE	+	12 _a	13 _b 25 _k
	-	2 _c	23 _d 25 _l
<hr/>			
		14 _m	36 _n 50

$$\begin{aligned}
 \text{The phi coefficient, } \phi &= \frac{ad - bc}{\sqrt{klmn}} \\
 &= \frac{12 \cdot 23 - 2 \cdot 13}{\sqrt{25 \cdot 25 \cdot 14 \cdot 36}} = \frac{276 - 26}{25 \sqrt{504}} \\
 &= \frac{250}{25 \sqrt{504}} \\
 &= \underline{\underline{0.4454}}
 \end{aligned}$$

The size of the coefficient is related to the way in which the two variables are split. When both variables are evenly divided the maximum limits of a correlation coefficient ± 1 are obtained. If the marginal totals are unequal, the maximum values vary, but in any case are less than ± 1 . For the division of the variables in

this sample the maximum value of the phi coefficient is calculated below:

		RECOVERY OF <u>MUCOSALIS</u>		
		+	-	
DEMONSTRATION OF MUCOSALIS BY IMMUNOFLUORESCENCE	+	25	0	25
	-	11	14	25
		36	14	50

$$\begin{aligned}
 \text{Maximum } \Phi &= \frac{25 \cdot 14 - 11 \cdot 0}{\sqrt{25 \cdot 25 \cdot 36 \cdot 14}} = \frac{350}{25 \sqrt{504}} \\
 &= \underline{\underline{0.6236}}
 \end{aligned}$$

From this it can be seen that taking into account the limitations of the techniques the correlation between the recovery of mucosalis bacteriologically and its demonstration by immunofluorescence is reasonable.

Summary of Observations made on Necropsied Pigs (Tables 4s to 4x)

Abbreviations used in Tables 4s to 4x.

Pathology/Histology - N.A.D. = No abnormality detected

Experimental Status - D. = Dosed

N.D. = Not Dosed

Immunofluorescence - +/- = A little specific fluorescence, but not as bright as in the positive controls and only in some cells per gland.
 - ++/-- = As bright as in the positive controls, but not a very large amount of fluorescence.
 - ++/++ = Fluorescence present in considerable amounts and as bright as in the controls.

DISCUSSION

In the experiments described in this chapter, infection with mucosalis in both the mouth and intestinal tract of piglets, dosed orally with mucosalis in the neonatal period, has been established. This was achieved without recourse to other treatment of the piglets, except for the administration of chalk to neutralize the gastric acidity. The litter of sow B did not receive chalk before dosing and mucosalis still became established, albeit in only a small proportion of the litter. It is not possible to assess the significance of the failure to give chalk to the litter of sow B, as the number of piglets is only small. The pH in the stomach of the young piglet is higher than in the adult, and in the very young piglet remains close to the diet (Manners, 1976). Usually the milk has a large buffering capacity, but the piglets in this litter were dosed before suckling and so pH may have been lower. Smith and Jones (1963) have reported that the pH of the piglet's stomach is much higher in the first day of life. Knop and Rowley (1975b), in experiments with Vibrio cholerae in infant mice, showed that the population of viable Vibrio cholerae was not reduced in viability on passing through the stomach, in contrast to the situation in adult mice.

This susceptibility of the neonatal pig to mucosalis infection is in contrast to the situation in the post-weaned pig (see Chapter V). In considering this there are a number of possible reasons. As discussed in the introduction, the intestinal epithelial cell of the neonate has a specialised apical endocytic complex for the uptake of particulate material, including virus particles. This is one of the

suggested reasons for the greater severity of TGE virus infection (Wagner, Beamer and Ristic, 1973), and of reo-like virus infection (Lecce, King and Mock, 1976) in neonatal piglets; this specialisation of the epithelial cell allowing more virus to enter the cell. Uptake of E. coli by the intestinal epithelium of the neonatal pig has also been described (Staley, Jones and Corley, 1969d), but the circumstances of the experiment were very artificial, using germ-free colostrum deprived piglets exposed to very large numbers of bacteria. Since mucosalis is located in an intracellular site in PIA (Rowland, Lawson and Maxwell, 1973; Lawson and Rowland, 1974), this specialisation of the intestinal epithelial cell may be important in the entry or uptake of this organism into the cell.

Also in the neonatal pig the cell kinetics of the intestinal epithelium differ from that of the older, post-weaned pig (Moon, 1971). The replacement time for the jejunal and ileal epithelium is from seven to ten days in the one day old pig, and two to four days in the three week old pig. This is also true for the large intestine where the life span of the epithelial cells in the caecum and colon of the one day old pig is four to five days, and in the three week old pig two to three days. These figures are for suckling pigs; after weaning the villous atrophy and crypt hyperplasia which occur (Kenworthy, 1976) probably indicate an even greater rate of loss. This may be important with mucosalis for if it cannot establish before the epithelium is replaced persistent infection will not occur. One can envisage a delicate balance between the rate of division of mucosalis and the rate of loss of the intestinal epithelium.

So that if the epithelial cell life span is short, mucosalis may not be able to divide at a sufficient rate to establish, and is instead shed with the epithelial cells, and vice versa. The increased life of the intestinal epithelium in the neonate may mean that mucosalis has a greater chance of establishing itself.

Whether or not the piglets had received colostrum before oral dosing with mucosalis did not seem to affect their susceptibility to mucosalis infection. The site of infection did, however, vary, depending on whether dosing with mucosalis was carried out before or after suckling. In those piglets dosed before suckling the isolations of mucosalis were all from the large intestinal mucosa, except for one and that piglet had not been orally dosed. From this latter piglet mucosalis was recovered from the terminal small intestine. In those piglets dosed after suckling most of the recoveries of mucosalis were made from the small intestine. The intestinal absorptive cells of the pig duodenum, and to a lesser extent the jejunum, but not the ileum, undergo a cytological maturation after exposure to colostrum (Staley, 1969). In the light of these results, it is tempting to suggest that entry or uptake of mucosalis by the epithelium is stimulated in the presence of colostrum, so that if the pigs are exposed to mucosalis after suckling infection will be predominantly in the upper small intestine. If they are dosed with mucosalis before suckling, peristalsis may move the inoculum down towards the large intestine, before colostrum can have an effect on entry into the small intestinal epithelium. The pinocytotic and phagocytic activity of macrophages is affected by a wide range of

substances, and the situation for mucosalis and the intestinal epithelium may be similar.

However, if uptake is by a mechanism such as this, mucosalis would be expected to lie within a vacuole, as described for E. coli (Staley, Jones and Corley, 1969d). In the work of Staley et al., large enough numbers of bacteria were present for electron microscopy to be rewarding, in contrast to the low level of infection achieved here. However, as already discussed the circumstances of the experiments of Staley et al., were very artificial. Escape from intracellular vacuoles, after uptake, is thought to occur with some rickettsia (Weiss, 1973) and Trypanosoma cruzi (Tanowitz et al., 1975). The fact that mucosalis is usually free in the intestinal epithelial cell cytoplasm does not preclude uptake by a similar mechanism.

In the litters dosed after suckling inclusions were seen within the intestinal epithelial cells. Staley, Jones and Corley (1969c) suggested, that similar structures which they observed, were bilirubin crystals formed from the breakdown of ingested red blood cells. Hardy, Hockaday and Tapp (1970) were of the opinion that the inclusions were formed by the condensation of vacuoles seen at an earlier stage and which contained light flocculent material. No attempt was made to further examine or identify the inclusions seen in this work.

Another effect of colostrum which it was thought might operate was a protective one, to specifically or non-specifically prevent mucosalis infection. Colostral immunity to TGE virus (Bohl and Saif, 1975) and E. coli (Rutter et al., 1970) in the pig, has been described. In man, after oral infection with E. coli specific

colostral antibody appeared and colostrum cells producing secretory IgA against that organism (Goldblum et al., 1975). It has been suggested that this is due to the migration of lymphoid cells, which are stimulated by antigen taken up from the gut, migrating to distant secretory surfaces (Williams and Gowans, 1975). The sows, whose litters were used in these experiments were mostly obtained from a farm (A) on which PIA occurs, and the mucosalis strain used in these experiments (106/75) was originally recovered from a pig on that farm. So, it was possible that the sows had been exposed to mucosalis and had a degree of colostrum immunity.

In the litters dosed after suckling mucosalis was recovered from a larger proportion of the undosed piglets (2/3) than in the litters dosed before suckling (1/8). The spread from the dosed to the undosed piglets was rapid, recovery of mucosalis being made from the M.S.I. contents at twenty four hours, and the T.S.I. mucosa at forty eight hours, in undosed piglets. Spread presumably was by coprophagy or mouth to mouth by way of the teats.

Pharmacologically mediated hypomotility to reduce peristalsis is important in establishing mucosalis infection in the post-weaned pig (Chapter V). It is more difficult to assess its effect in these experiments. Only the litter of sow J received benzetimide, and although this was the only litter in which definite adenomatous change was shown and mucosalis demonstrated in an intracellular site, mucosalis was only recovered from one pig. These piglets were not dosed until three days of age, the oldest litter in this series of experiments. So the role of benzetimide in this age group is unresolved, but in the transmission experiments in neonatal piglets it

was found to be very useful (Chapter VII). The overall passage time is slower in the suckling piglet compared to the weaned piglet (Kidder and Manners, 1974). This could in itself be one of the reasons for the greater susceptibility of the neonatal piglet to mucosalis when compared to the post-weaned pig.

More piglets died after dosing in the pre-suckled experiments as would be expected if one delayed the acquisition of both the nutritive content and the protective factors in colostrum. Mucosalis was only recovered from one of these piglets. In that piglet it was isolated in large numbers from the large intestinal contents, the large intestine itself being grossly distended. The post-mortem findings were unusual, but since it occurred on only one occasion there is insufficient evidence to link mucosalis with the changes observed.

From the litter of sow C no recoveries of mucosalis were made, there were no clinical signs, gross or histological changes and no specific fluorescence with mucosalis anti-sera. The suggestion from this is that some piglets are possibly not susceptible to mucosalis infection, under the circumstances pertaining in these experiments. Whether or not those piglets in the other litters, in which mucosalis was not demonstrated were also not infected is unknown. Even in those piglets from which mucosalis was recovered, the numbers isolated were low, and if adenomatous glands were seen, they were few in number and only present in some sections. The chances of missing such glands or failing to recover mucosalis under such circumstances were quite high, when one considers how small a portion of the alimentary tract one can examine from each animal. In connection with

the susceptibility to infection a genetic resistance to intestinal infection with E. coli has been described (Sellwood et al., 1975), related to the absence on the intestinal epithelial cells of a receptor for the bacterial K88 antigen. This receptor specificity and attachment may be important in mucosalis infection. Although located in an intracellular site rather than on the mucosal surface in the intestine, attachment may be a necessary prerequisite for internalization. Such susceptibility may be related to receptors with blood group reactive substances (Williams and Gibbons, 1975). It could also provide a partial explanation for the occurrence of PIA in some members of a litter only, and in only some litters on any premises, as is seen with the natural disease.

There was a reasonable correlation between the recovery of mucosalis from any piglet, and the demonstration of mucosalis antigen by immunofluorescence. Mucosalis was recovered from the piglets in these experiments up to forty days post-dosing, and the presence of specific fluorescence in the intestinal mucosa mostly paralleled this. Occasionally the fluorescence was detected for a short time after the recovery of mucosalis was no longer achieved. The presence of specific particulate fluorescence within the lamina propria of the intestine has not previously been recorded. It is thought that the antigen was probably located within macrophages, although this could not be confirmed. During the recovery phase of PIA and immediately afterwards, mucosalis can be seen ultrastructurally within macrophages in the lamina propria (Chapter IX). At that time there is also specific particulate fluorescence, resembling that seen here, within

macrophages in the lamina propria. In experimentally produced PIA (Chapter VII), this was seen from fifty three days post-dosing. The fluorescence here was seen at a much earlier stage post-exposure.

Attention has already been drawn to a number of the technical difficulties in a study of this kind. When dealing with a low level of mucosalis infection these difficulties are exacerbated. The selective medium, N.B.G., was found to be of great value, but filtration less so under the circumstances of these experiments. This may have been due to the fact filtration reduced the already low levels of mucosalis to a level at which they could no longer be recovered. Also, if mucosalis is cell associated it may not be filterable at 0.8 μ . Only a limited portion of the alimentary tract was examined bacteriologically and similarly with histology, immunofluorescence and electron microscopy only a small amount of the total alimentary tract was examined.

To summarise, these experiments have shown that the neonatal piglet is susceptible to mucosalis infection after oral dosing. Mucosalis establishes in both the mouth and intestine. This is true whether the piglets are allowed to suckle before dosing or not, although differences in the distribution of mucosalis may depend on this. Under normal circumstances only a small number of organisms establish, and spread takes place rapidly to undosed litter mates. Mucosalis can be recovered from the mouth and intestine for up to forty days post-dosing, and the demonstration of mucosalis antigen by immunofluorescence parallels this. The correlation between the

demonstration by the fluorescent antibody technique and bacteriological recovery was good. Possible factors responsible for this susceptibility of the neonatal piglet are discussed, and also technical difficulties inherent in the experiments.

CHAPTER V

INFECTIVITY EXPERIMENTS USING POST-WEANED PIGS, WITH
SPECIAL REFERENCE TO PHARMACOLOGICALLY INDUCED HYPOMOTILITYINTRODUCTION

PIA is clinically recognised as a condition of the post-weaned pig, which is most often encountered in the six to fifteen weeks old age group (Rowland and Lawson, 1974). It is also reported in older pigs (Moynihan and Gwatkin, 1941), including pigs of bacon weight at slaughter (Emsbo, 1951; Hoorens, 1962; Westendorp, 1965). RI and NE are similarly recognised as conditions of the post-weaned pig (Veterinary Investigation Service, 1959 and 1960). Digestive disturbances in pigs post weaning are well documented, and Kenworthy (1976) described an inflammatory response in the gut after weaning. Kenworthy and Allen (1966) considered that the role of coliform bacteria is secondary to the change in diet in colibacillosis. There is an increase in the metabolic activity of the gut microflora associated with the change in diet at weaning (Porter and Kenworthy, 1969), this may be involved in the associated degenerative changes seen in the epithelial cells (Kenworthy, Stubbs and Syme, 1967). Enteric disease contributes significantly to economic loss in this age of pig (Veterinary Investigation Service, 1960). Around weaning litters of pigs are often mixed, which may result in spread of pathogens between individuals, and alterations of the gut microbial flora at this time (Lee and Gemmell, 1972). Stress also, either environmental or dietary, is associated with alterations in the gastro-intestinal flora (Tannock and Savage, 1974). As PIA, RI and NE occur clinically in the post-weaned pig, this category of piglet was used in the infection experiments described in this chapter.

Initial immunity in the suckling pig is colostrum-derived and these serum antibodies decline with age, leaving a postulated period of critical antibody deficiency in piglets at three weeks of age, (Brown et al. 1961; Miller et al. 1962). Piglets of this age were used in some of the experiments. In the suckling pig maternal secretory IgA, in colostrum and milk has a local protective effect in the intestinal tract which may be sustained until weaning (Porter, 1973). Production of IgM, by the piglet gut precedes that of IgA. (Allen and Porter, 1973 and 1977), and IgM, cells form the majority of immunoglobulin containing cells in the lamina propria of the small bowel in suckling pigs up to four weeks of age, with the proportion of IgM, cells declining with maturity (Allen and Porter, 1977). Synthesis of IgM, is possible from late in the first week of life and secretion of IgA, can be detected at about the tenth day (Porter, 1973). At weaning the protective effect of the IgA, in milk is lost, and the level of intestinal immunity from locally produced immunoglobulins may not be protective. For these reasons one might expect this age of piglet to be susceptible to enteric infection with mucosalis.

The resident gastro-intestinal flora is thought to have a protective function, by operating to prevent establishment of invading pathogens in the alimentary tract. Inhibition of this flora by administration of antibacterial drugs can allow experimental intestinal infection with enteric pathogens under certain circumstances. For example pretreatment of mice with streptomycin reduced the infective dose of Salmonella enteritidis from one million organisms to one (Bohnhoff and Miller, 1962). A number of piglets in this study were pretreated with oral streptomycin prior to exposure.

The effect of pharmacologically induced gasto-intestinal hypomotility on the establishment of mucosalis infection in post-weaned pigs was also examined using benzetimide, an anti-cholinergic agent which reduces gasto-intestinal peristalsis (Marsboom, Temmerman and Symoens, 1973).

These experiments are divided into two parts; part A. deals with ten to fourteen day old piglets and part B. with five week old piglets.

PART A. TEN TO FOURTEEN DAY OLD PIGLETSMATERIALS AND METHODSExperimental Animals

The piglets used in these experiments were obtained from two sources; herds B and D. The details of the piglets are summarised in table 5a.

TABLE 5a DETAILS OF EXPERIMENTAL ANIMALS

Experi- ment	Age of Piglets at weaning	Age of Piglets at dosing	Age of Piglets at necropsy	Source	Piglet Ref.
I	11 days	11 days and 13 days	14 days	Herd D	223/74
	11 days	11 days and 13 days	14 days	Herd D	229/74
	11 days	11 days and 13 days	16 days	Herd D	230/74
II	10 days	11 days	18 days	Herd B	243/74
	10 days	11 days	22 days	Herd B	245/74
	10 days	11 days	25 days	Herd B	252/74
	10 days	Not dosed	23 days	Herd B	243/74
III	14 days	18 days	22 days	Herd B	286/74
	14 days	18 days	25 days	Herd B	288/74
	14 days	18 days	29 days	Herd B	292/74
	14 days	Not dosed	26 days	Herd B	289/74

Infecting Organism

Strain 604/74 L1-3 of C. sputorum subsp. mucosalis was used for oral inoculation in these experiments. This had been isolated from the large intestine of a fifteen week old pig, which had lesions of PIA involving the large bowel. At the time the experiments were carried out it was the most recent isolate of mucosalis available.

Numbers of viable Mucosalis (604/74 L1-3) received by the piglets:-

Experiment I 228/74, 229/74 and 230/74 received 4.75×10^{10} and 2.3×10^{10}
 Experiment II 243/74, 245/74 and 252/74 received 5.0×10^{11}
 Experiment III 286/74, 288/74 and 292/74 received 2.25×10^{10}

Streptomycin

The streptomycin used to orally dose the piglets in experiment III was a four-fold dilution of 'Dimycin' (Glaxo Laboratories, Greenford, Middlesex) in 0.1M. pH. 7.2 P.B.S. Each millilitre of this four-fold dilution of 'Dimycin' contained 33.4 mgs. of streptomycin and 33.4 mgs. of dihydrostreptomycin, both present as sulphates. The four piglets received five mls. of this on two separate occasions.

Creep-feed

It was discovered in retrospect that the creep-feed to the piglets in these experiments was medicated with antibiotic. It contained twenty grams per ton of Eskalin 500 (10 Megaunits of Virginiamycin). Media was prepared by Dr. G.H.K. Lawson (Department of Veterinary Pathology, Edinburgh University), containing an equivalent concentration of virginiamycin, to determine its effect, if any, on mucosalis. It appeared that, at the levels present in the creep, virginiamycin had no effect on the growth of mucosalis, in vitro. A number of strains, including the

inoculating strain, grew on media containing virginiamycin at the concentration used in feed (4 units/ml.). For ten out of ten strains tested the minimal inhibitory concentration was >5 units/ml. and for nine out of ten strains was >25 units/ml. Levels in the intestinal contents and tissues were almost certainly less than the 4 units/ml. in which it was present in the feed.

Management of Piglets

The piglets were introduced individually into pens built from straw bales, in freshly formalin fumigated rooms in an isolation block. Heat was provided from overhead lamps. The control piglets, which were not dosed with mucosalis, were housed in separate rooms. Management procedure was such as to minimise the risk of spread of organisms from the dosed to the control pigs.

Dried Milk, S.M.A. (John Wyeth and Brother Ltd., Berks.), made up to the manufacturer's recommendations, was offered twice daily to the piglets, and any remaining from the previous feed was discarded. The piglets also received creep pellets (406 creep feed pellets, B.O.C.M. Silcock, Basingstoke, Hampshire) and water ad-lib.

Timing of Procedures

Expt. I

The piglets 228/74, 229/74 and 230/74 were orally dosed at eleven and thirteen days of age, i.e. at weaning and forty eight hours post-weaning. The piglets 228/74 and 229/74 were found dead twenty four hours after receiving the second inoculum of mucosalis, and the third 230/74 was found dead three days after this second inoculum. Necropsies were carried out on these piglets at that time.

Expt. II

Piglets 243/74, 245/74 and 252/74 were dosed orally with mucosalis at eleven days of age, twenty four hours post-weaning. Piglet 243/74 was killed at seven days post-dosing, piglet 245/74 at eleven days post-dosing, and piglet 252/74 at fourteen days post-dosing. The uninoculated piglet was killed at twenty three days of age.

Expt. III

Piglets 286/74, 288/74, 292/74 and 289/74 were weaned at fourteen days of age. Two days post-weaning they received streptomycin orally, given in the morning and in the evening. They were dosed orally with mucosalis forty hours later at eighteen days of age, i.e. four days post-weaning. Oral swabs were taken from each piglet before, at forty eight hours and four days after dosing. Piglet 286/74 was killed at four days post dosing. Oral swabs were taken at seven days and piglet 283/74 was killed at that time. The control piglet 289/74 was killed at twenty six days of age. An oral swab was taken from the remaining piglet 292/74 at nine days post dosing and this piglet was killed at eleven days.

Sites ExaminedExpt. I

The small and large intestinal contents only, were examined bacteriologically from piglets 228/74, 229/74 and 230/74 which died. Tissue from the M.S.I. and L.I. of these piglets was fixed for histological examination only.

Expt. II

From piglets 243/74, 245/74, 248/74 and 252/74 the mucosa and contents from sites 1, 2, 3, 4 and 5 (Chapter II) were examined bacteriologically.

Expt. III

The mucosa from sites 1, 2, 3, 4 and 5 was examined bacteriologically in piglets 286/74, 288/74, 289/74 and 292/74. From the eight pigs in experiments II and III tissues from the five sites were also prepared for histological, immunofluorescence and electron microscopic examination.

Bacteriological Examination

The contents of the small intestines and large intestines of the piglets in experiment I were plated out onto S.B.A. and McConkey plates which were incubated aerobically at 37°C., and onto C.B.A. and N.B.G. plates which were incubated microaerophilically at 37°C.

Samples from the piglets in experiments II and III were examined as indicated in table 5b. In addition to this in experiment II filtered mucosal samples were also examined. For all five sites the last three or four drops of filtrate from the filtered mucosa were inoculated onto N.B.G. and C.B.A.

TABLE 5b

BACTERIOLOGICAL EXAMINATION - EXPERIMENTS II and III

Experiment	Sample	Sites	Dilutions Spread on N.B.G.	Dilutions Spread on C.B.A.
II	Unfiltered Mucosa	1,2 and 3	$\frac{1}{20}, (\frac{1}{20})^2$	$\frac{1}{20}, (\frac{1}{20})^2, (\frac{1}{20})^3, (\frac{1}{20})^4$
	Unfiltered Mucosa	4 and 5	$\frac{1}{20}, (\frac{1}{20})^2, (\frac{1}{20})^3$	$\frac{1}{20}, (\frac{1}{20})^2, (\frac{1}{20})^3, (\frac{1}{20})^4$
	Contents*	1,2,3,4 & 5	$(\frac{1}{10})^2, (\frac{1}{10})^3, (\frac{1}{10})^4$	$(\frac{1}{10})^3, (\frac{1}{10})^4, (\frac{1}{10})^5, (\frac{1}{10})^6$
III	Unfiltered Mucosa	1, 2 and 3	$(\frac{1}{20})^2$	$(\frac{1}{20})^2, (\frac{1}{20})^3, (\frac{1}{20})^4$
	Unfiltered Mucosa	4 and 5	$(\frac{1}{20})^2, (\frac{1}{20})^3$	$(\frac{1}{20})^2, (\frac{1}{20})^3, (\frac{1}{20})^4$

- * In addition the contents of sites 2 and 3 were spread onto N.B.G. + N. plates at the $(\frac{1}{10})^2$ dilution, and the contents for sites 4 and 5 at the $(\frac{1}{10})^2$ and $(\frac{1}{10})^3$ dilutions.

Results

Clinical Findings/Daily Observations

Expt. I

Piglets 228/74, 229/74 and 230/74 drank the milk offered from the time of weaning, and appeared alert and bright. 228/74 and 229/74 were found dead, with no previous clinical signs, at three days post-weaning i.e. three days and twenty four hours post oral dosing with mucosalis. 330/74 was alert and bright up to four days post-weaning and at this time although still drinking, there was evidence of scouring. This latter piglet was found dead five days post-weaning.

Expt. II

The undosed control piglet 248/74 remained healthy, drank the milk offered to it and took creep pellets throughout the duration of the experiment. At the beginning of the experiment the body weight of pig 248/74 was 6.16 lbs. and it had gained 3 lbs at euthanasia, on day 12 (day 1 is the day of oral dosing with mucosalis). Piglet 252/74 remained well during the experiment, weight at dosing was 8.36 lbs., and this had increased by 3.75 lbs., at euthanasia on day 14. Piglets 243/74 and 245/74 became diarrhoeic on day 2 lasting until days 4 and 5 respectively; the faeces varied from grey with mucus to yellow and fluid during this period. Both piglets were orally dosed with glucose saline at this time and were clinically normal at euthanasia on days 7 and 11 respectively, when they weighed 0 lbs., and 1.3 lbs. more than their weights on day 1.

Expt. III

At weaning piglet 289/74 weighed 9.35 lbs. and it had remained clinically normal and had gained 2.2 lbs. at euthanasia twelve days post-weaning. Piglet 286/76 was clinically normal until day 2 (day 1 was the day of oral dosing) when it became depressed and diarrhoeic. On day 3 it was brighter but still diarrhoeic, however on day 4 at euthanasia there was no evidence of scouring. At weaning piglet 286/74 weighed 8.8 lbs. and its body weight did not change during the experiment. Piglets 288/74 and 292/74 remained clinically normal throughout the experiment, eating and drinking greedily. At weaning their body weights were 11.55 lbs. and 13.2 lbs. respectively and they gained 3.35 lbs. and 2.2 lbs. during the experiment.

Pathological ResultsGross FindingsExpt. I Pigs 228/74, 229/74 and 230/74

These three piglets showed similar gross post-mortem changes and are therefore considered together. The carcasses were dehydrated, the stomach contained some clotted milk and the mucosa was congested, the small and large intestines contained varying amounts of fluid and the mucosa was congested. The findings could not be differentiated from those seen in enteric colibacillosis.

Expt. II Pigs 243/74, 245/74, 248/74 and 252/74

No gross lesions were detected in any of these piglets at necropsy.

Expt. III Pig 289/74

At necropsy no gross lesions were found in the alimentary tract. The right apical lobe of the lung was firm, consolidated and red-grey in colour. No abnormalities were detected in the remainder of the carcass.

Pig 286/74

At necropsy there were no gross lesions detected in this pig.

Pigs 288/74 and 292/74

No abnormalities were seen in the alimentary tract of these pigs at necropsy. The lungs of both piglets were firm, consolidated and red-grey in colour. In pig 288/74 the ventral dependant areas of the apical lobes, and in pig 292/74 the ventral areas of the apical and cardiac lobes were involved.

HistopathologyExpt. I Pigs 228/74, 229/74 and 230/74

There was post-mortem autolysis of the intestinal mucosa of these piglets, but the morphology could still be discerned. There was a marked congestion of the blood vessels of the mucosa, but otherwise no abnormalities were seen.

Expt. II Pig 248/74 (Control)

In the small intestine (sites 1, 2 and 3) there was villous atrophy and crypt hyperplasia, but the epithelium otherwise appeared normal. The lymphoid follicles of Peyer's patches were enlarged and reactive. The epithelium of the large intestine (sites 4 and 5) was normal, but there was an increase in the cellularity of the lamina propria,

mainly due to lymphocytes and plasma cells. In all sites there was oedema of the lamina propria.

Pig 243/74

The histopathological findings in this pig were essentially the same as those in the undosed control pig 248/74.

Pig 245/74

In the small intestine (sites 1, 2 and 3) there was villous atrophy and crypt hyperplasia, which was greater than that in pigs 248/74 and 243/74. There were neutrophils present in moderate numbers in the lamina propria, and in some areas the lamina propria was oedematous. The lymphoid tissue of Peyer's patches was enlarged and the follicles had a reactive appearance. In the large intestine (sites 4 and 5) the epithelium appeared mainly normal but in some glands cellular debris was present in the lumen. There was an increase in the cellularity of the lamina propria, mostly due to cells of the lymphocytic series, but occasional neutrophils were also seen.

Pig 252/74

The intestinal changes were essentially the same as in pig 245/74. In the large intestine there was an increase in the cellularity of the lamina propria due predominantly to lymphocytes and plasma cells. In the ileo-caeco-colic lymph node there were a number of interesting features. The lymph nodes were hyperplastic with numerous reactive germinal centres present. More to the periphery of the lymph node there were numerous giant cells (fig. 57). Some of these were of foreign-body giant cell morphology, while others were of the Langhan's type. Zeihl-Neelsen stained sections failed to demonstrate mycobacteria, and similarly no information

was obtained with Giemsa, P.A.S. and P.B.R. staining. There was also a large number of eosinophils present in the lymph nodes in the peripheral and central sinuses.

Expt. III Pigs 286/74, 288/74, 292/74 and 289/74

In all the pigs in this experiment the intestinal changes were essentially similar. In the small intestine (sites 1, 2 and 3) there was villous atrophy and crypt hyperplasia. There was an increase in the cellularity of the lamina propria, a variety of cells being involved; plasma cells, macrophages, lymphocytes and some neutrophils. In the large intestine (sites 4 and 5) there was oedema of the lamina propria in some areas, and an increase in the cellularity as in the small intestine.

The histopathological changes in the lungs of pigs 288/74, 289/74 and 292/74 were of a bronchiolitis and an exudative pneumonia. The affected areas were congested, there were neutrophils in the alveoli, a proliferation of septal cells and swelling and degeneration of alveolar lining cells.

Electron-microscopy

A limited number of grids from some of these piglets were examined by E.M. No evidence of bacteria within the epithelial cells, or changes in the epithelial cells themselves were seen. In view of the limited specific fluorescence and the fact that mucosalis was only recovered from one piglet, and then from the oral cavity, too thorough an examination of tissues from the intestinal tracts of these piglets was not considered justified. The giant cells seen histologically could not be located at an ultrastructural level.

Bacteriological ResultsExpt. I Pigs 228/74, 229/74 and 230/74

Haemolytic E. coli was grown in almost pure culture from the intestinal contents of these three piglets, both aerobically and micro-aerophilically. The serotype isolated was O149 : K91, K88 ac. Mucosalis was not recovered from any of the three piglets.

Expt. II Pig 243/74, 245/74, 248/74 and 252/74

Mucosalis was not recovered from the three piglets killed at seven, eleven and fourteen days post dosing with mucosalis, or from the undosed piglet, at euthanasia.

Expt. III Pig 289/74 (not dosed with mucosalis)

Oral swabs from this piglet taken four and seven days after oral dosing of the other three piglets in this group with mucosalis, were negative for mucosalis. At euthanasia mucosalis was not recovered from the mucosa at any of the five sites examined or from the oral cavity.

Pig 286/74 (Euthanasia four days post dosing with mucosalis)

At two days post-dosing a rectal swab from this piglet yielded large numbers of the enteropathogenic E. coli serotype O149 : K91, K88 ac. At euthanasia mucosalis was not recovered from the mucosa at any of the five sites examined. Mucosalis was however isolated from a mouth swab taken at that time.

Pig 288/74 (Euthanasia seven days post-dosing with mucosalis)

An oral swab taken from this piglet four days post dosing was negative for mucosalis. At euthanasia mucosalis was neither recovered from the alimentary tract mucosa at any of the five sites examined, nor from the mouth.

Pig 292/74 (Euthanasia eleven days post dosing with mucosalis)

Mucosalis was not isolated from mouth swabs taken from this piglet at four days, seven days and nine days post dosing, or at euthanasia.

Mucosalis was not recovered from the mucosa at the five sites in the intestine examined, at necropsy.

Bacterial growth on the plates was such that mucosalis, if present should have been recognisable. Occasionally the C.B.A. plates at the lower dilutions of the large intestinal mucosa, and of the contents of both the small and large intestines were overgrown, but the growth on the corresponding N.B.G. plates should have allowed recognition of any mucosalis colonies present.

Campylobacter coli was isolated on a number of occasions, an indication that if mucosalis had been present in similar numbers and viable, it too should have been recovered. The filtered mucosal samples were less contaminated than the equivalent unfiltered samples. On occasions C. coli was isolated from the filtered mucosal samples and not from the corresponding unfiltered sample.

Immunofluorescence Results

Expt. I Pigs 228/74, 229/74 and 230/74

Immunofluorescence staining was not carried out on tissues from these pigs.

Expt. II Pigs 243/74, 245/74, 248/74 and 252/74

Tissues from all five sites in the intestines of these pigs, and also from the mesenteric lymph nodes were examined. A cryostat section was stained by H. and E. to ascertain the morphology of the tissue, and

to ensure that representative areas of crypts and villi were present. In no case was there fluorescence comparable to that in the known positive controls stained at the same time. The negative control sections did not show any specific fluorescence, only autofluorescence by eosinophils, which was dull yellow and easily recognised. Occasionally the mucus in goblet cells showed a dull fluorescence, but this could be readily differentiated using an alternative exciting source (HB200-UV). The piglet which had not been dosed, pig 248/74, was negative. In the other three piglets, and especially in piglet 245/74, in occasional glands there was some apparently specific particulate fluorescence. Mostly this appeared to be in the epithelium, but was also seen in the lamina propria. It must be stressed however that this was only present in small amounts in occasional glands. It appeared to be mostly within the deeper crypt areas of the mucosa. There were small amounts of apparently specific fluorescence of very occasional cells in the mesenteric lymph nodes, but it was difficult to be certain due to the autofluorescence of many of the cells present.

Expt. III Pigs 286/74, 288/74, 289/74 and 292/74

In the pig which had not been dosed with mucorale there was no specific fluorescence seen. In the other three pigs very occasional cells in some glands, especially in the crypts, showed some specific particulate fluorescence. Again it must be emphasised that the amount of fluorescence was small, but that it did not appear to be present in the undosed animal.

PART B. FIVE WEEK OLD PIGLETS WITH SPECIAL REFERENCE TO
PHARMACOLOGICALLY INDUCED HYPOMOTILITY

MATERIALS AND METHODS

Experimental Animals and timing of procedures

The piglets used in this experiment were obtained in two groups from farm B (Chapter II). These piglets had been on feed trials and had been housed individually in metabolism cages. They were five weeks old. They were introduced into recently formalin fumigated rooms in an isolation block. Each group of six animals was housed together. The piglets in each group were dosed according to the regime in table 50.

Infecting Organism

The strain of C. sputorum subapp. mucosalis 106/75 Sl. 10^{-3} was used in these experiments.

Benzetimide

The piglets were dosed with Benzetimide at the rate of 0.250mg/kg. body weight (Mareboom et al., 1973). The Benzetimide solution was stored at 4°C. The required amount of benzetimide solution was made up to 10 mls. in sterile distilled water and the pigs were dosed orally using a short intra-oesophageal tube and a syringe.

Management of piglets

The piglets were observed at least once daily and received meal (A.B.R.O Breeders - Seafield Mill, Roslin) and water ad-lib.

TABLE 5C

EXPERIMENTAL ANIMALS AND TIMING OF PROCEDURES

Piglet Reference	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day of Euthanasia
<u>Group 1</u>							
315/75	B	M ¹		B	M ²	M ³	13
321/75	B	M		B	M	M	14
312/75		M			M	M	12
320/75		M			M	M	14
314/75	B			B			13
319/75							14
<u>Group 2</u>							
361/75	B	M ⁴	M ⁵		B	M ⁶	13
366/75	B	M	M		B	M	15
360/75		M	M			M	13
363/75		M	M			M	14
362/75	B				B		14
367/75	,	,	,	,	,	,	15

B = Dosed with benzetimide

M = Dosed with mucosalis

1. 1.375×10^{10} viable mucosalis strain 106/75
2. 2.2×10^{10} " " " "
3. 1.1×10^{10} " " " "
4. 2.8×10^{10} " " " "
5. 5.6×10^{10} " " " "
6. 3.0×10^{10} " " " "

Sites Examined

The mouth, and the mucosa of the M.S.I., T.S.I. and L.I. were examined bacteriologically in all pigs. Tissues from the M.S.I., T.S.I. and L.I. were fixed for histological, immunofluorescence and electron microscopic examination.

Bacteriological Examination

The mouth swabs were dealt with as described in Chapter II. The unfiltered mucosal samples from all three sites were spread onto B.A. and N.B.G. plates at the $1/20$, $(1/20)^2$ and $(1/20)^3$ dilutions. The filtered mucosal samples, prepared in P.B.S. were spread onto B.A. and N.B.G. plates at the $1/10$ dilution.

RESULTS

Clinical Signs/Daily Observations

Throughout the duration of the experiments, all the piglets remained alert, bright and had good appetites. There was no evidence of scouring.

Pathological Results

Gross Findings

All the pigs were well grown, and no gross lesions were detected in any organs of the body. The mucosa of all regions was unremarkable, Peyer's patches were clearly visible in the terminal ileum but the mucosa appeared normal to the naked eye. The mesenteric lymph nodes were solid, cellular and off white in colour, with an architectural pattern clearly visible on the cut surface.

HistopathologyPigs not dosed (319/75 and 367/75)

Histologically in the small intestines of these pigs there was a villous atrophy and crypt hyperplasia, and in some areas the lamina propria was oedematous. In the large intestine, in some areas there was slight surface damage and occasional neutrophils were seen in the lamina propria.

Pigs dosed with benzetimide (314/75 and 362/75)

The findings in these two piglets were as for the undosed piglet except that there was a greater number of eosinophils in the lamina propria of the terminal small intestine of pig 314/75.

Pigs dosed with mucosalis only (312/75, 320/75, 360/75 and 367/75)

These piglets showed villous atrophy and crypt hyperplasia in the small intestine, in which features they did not differ from those above. The lamina propria contained large numbers of cells of the lymphocytic series.

Pigs dosed with benzetimide and mucosalis (315/75, 321/75, 361/75 and 366/75)

The M.S.I. and T.S.I. in these four pigs showed villous atrophy and crypt hyperplasia. The large intestine in pigs 361/75 and 366/75 was unremarkable, but in pigs 315/75 and 321/75 the mucosa appeared to be increased in width in some areas, although it was not adenomatous. Some of the glands appeared enlarged, and had a crowded appearance, but were not definitely adenomatous. In some gland lumina there was cell debris.

Bacteriological Results

The bacteriological results are summarised in table 5d. From this it can be seen that approximately fourteen days after dosing with benzetimide and mucosalis it was possible, in three out of four pigs, to recover mucosalis. In two of the animals mucosalis was recovered from both the T.S.I. mucosa and L.I. mucosa. In the other groups; those receiving mucosalis only, those receiving benzetimide only, and those not dosed, mucosalis was not recovered from the alimentary tract at the end of a comparable period. Mucosalis was not recovered from the oral cavity of any of these piglets. The numbers of mucosalis isolated from the three pigs, along with other relevant details are shown in table 5d. From this it can be seen that four isolations were made from filtered mucosal samples, and two isolations were made from unfiltered samples. In only one case was mucosalis recovered from both the filtered and unfiltered samples of a given site. In four cases the isolations were made on non-inhibitory media, B.A., whilst in two cases isolations were made on inhibitory media, N.B.G. In the instances where recovery was made on N.B.G. media the corresponding B.A. plates had a much heavier growth, making recognition or recovery of mucosalis impossible or very difficult.

Table 5d

BACTERIOLOGICAL RESULTS

Status	Pig Reference	Sites from which <u>Mucosalis</u> recovered			Media on which, and sample from which <u>Mucosalis</u> was recovered.	
		Mouth	M.S.I.	T.S.I.	I.I.	
GROUP 1						
Dosed with <u>mucosalis</u> and benzetimide	315/75	-	-	+ ¹	+ ²	N.B.G. 1/20 dilution T.S.I. unfiltered Mucosa B.A. 1/10 dilution I.I. filtered Mucosa
	321/75	-	-	+ ³	+ ⁴	B.A. 1/10 dilution T.S.I. filtered Mucosa N.B.G. 1/10 dilution I.I. filtered Mucosa
Dosed with <u>mucosalis</u> only	312/75 320/75	- -	- -	- -	- -	
Dosed with benzetimide only	314/75	-	-	-	-	
Not dosed	319/75	-	-	-	-	
GROUP 2						
Dosed with <u>mucosalis</u> and benzetimide	361/75 366/75	- -	- -	- + ⁵	- -	B.A. 1/10 dilution T.S.I. filtered Mucosa B.A. (1/20) ² dilution T.S.I. unfiltered Mucosa
Dosed with <u>mucosalis</u> only	360/75 363/75	- -	- -	- -	- -	
Dosed with benzetimide only	362/75	-	-	-	-	
Not dosed	367/75	-	-	-	-	

viable mucosalis per gram of mucosa.

1.	5.0 x 10 ³	"	"	"	"
2.	> 2.0x10 ²	"	"	"	"
3.	> 1.0x10 ²	"	"	"	"
4.	> 2.2x10 ³	"	"	"	"
5.	8.0-10 ⁴	"	"	"	"

Immunofluorescent Results

Group 1 (Pigs 312/75; 314/75; 315/75 (+); 319/75; 320/75 and 321/75(+))

In none of the tissues from these pigs, i.e. M.S.I., T.S.I. and L.I. from each pig, examined by immunofluorescent staining was convincing positive specific fluorescence seen. There was some fluorescence but in comparison to the known positive controls it was of a very low order. Most of the fluorescence was present in the gland lumina and only in occasional glands. It was possible that some of this fluorescence was intracellular. In pigs 312/75 and 321/75 there was specific fluorescence in occasional cells in the lamina propria of the M.S.I. There was no difference between the six pigs with respect to the presence of this intraluminal fluorescence.

Group 2 (Pigs 360/75; 361/75; 362/75; 363/75; 366/75(+) and 367/75)

There was no specific fluorescence seen in these pigs using mucosalis anti-serum. In the lumen of very occasional glands there was some fluorescence, but as above no real significance can be attributed to this due to its limited magnitude and occurrence.

(+) = culturally positive for mucosalis.

Electron Microscopy

Tissues from a number of sites in these pigs were examined by electron microscopy. Particular attention was paid to those sites which had yielded mucosalis bacteriologically as it was thought that these might offer a better chance of success in locating the position of mucosalis. However in no case were intra-cellular bacteria seen in the intestinal epithelium. Similarly no significant changes were noted in the epithelial cells, the ultrastructure conforming to that described for the control pigs in chapter III, part A.

DISCUSSION

In these experiments it was not possible to establish demonstrable infection with mucosalis in the intestinal tract of "normal" post-weaned piglets. If one considers the difference in size of the alimentary tract of the post-weaned pig to that of the neonate, then the former will have a much greater intestinal surface area, and a very much larger number of epithelial cells in relation to the dose of mucosalis. The number of mucosalis per epithelial cell will therefore be much less in the post-weaned pig compared to the neonate. It is difficult to envisage how this dilution effect can be overcome without the administration of impossibly large volumes of mucosalis cultures. Attempts to alter the susceptibility of the post-weaned pig to mucosalis were therefore made.

Treatment with streptomycin in an attempt to alter the gastro-intestinal flora, and possibly enhance the establishment of mucosalis in the alimentary tract was unsuccessful, in contrast to its reported effect in salmonellosis (Miller and Bohnhoff, 1962 and 1963), shigella (Hentges and Freter, 1962) and vibrio (Freter, 1956) infections in laboratory animals. The gastro-intestinal flora is thought to manifest its inhibitory effect by the production of volatile fatty acids, especially butyric acid (Bohnhoff and Miller, 1962; Meynell, 1963; Lee and Gemmell, 1972). This attempt to alter susceptibility using streptomycin was only carried out in three piglets and so it is not possible to draw definite conclusions from the results. It is possible that if other conditions had been correct for the establishment of mucosalis then antibiotic pretreatment may have increased the numbers of mucosalis establishing or decreased the numbers of mucosalis necessary to establish infection. For these same reasons it is not possible to conclude that the normal gastro-intestinal flora does

not act in an antagonistic manner, thus preventing mucosalis becoming established. The micro-organisms in any such interaction, if one exists, may not be sensitive to streptomycin, or again the interactions may be more complex and although the antagonistic flora was removed or reduced the other conditions necessary for establishment of mucosalis may not have pertained.

These results indicate that hypomotility enhances the establishment of mucosalis in the alimentary tract. Peristalsis is recognised as one of the non-specific factors operating in a protective manner which prevents establishment of organisms in the alimentary tract (Dixon, 1960; Abrams, 1970; Savage, 1972). Reduced peristalsis has been reported to increase the susceptibility to a number of enteric organisms; salmonella (Takeuchi, 1967; Kent, Formal and Labrec, 1966), Shigella (Formal *et al.* 1963), and Vibrio cholerae (Knop and Rowley, 1975a). It is possible that the alteration in diet at weaning results in a decrease in gastro-intestinal peristaltic activity. It is known that after fasting only a relatively low flow of digesta persists (Noakes *et al.* 1967). Natural exposure to mucosalis at weaning may result in an enhanced chance of establishment of mucosalis in the alimentary tract. The situation around weaning is clearly complex and factors other than the number of mucosalis are important for the establishment of infection.

Spread of mucosalis within the groups of piglets in part B was not seen, in contrast to the situation which exists in the neonate (chapter IV), where there was rapid spread between dosed and undosed litter mates. The treatment of the pigs in the groups was such that some of the piglets were dosed with benzetimide and not mucosalis and none of these piglets which had been "conditioned" became demonstrably infected with mucosalis. The

numbers of piglets involved are too small for definite conclusions to be drawn on this latter point. The numbers of mucosalis passed in the faeces of this age of pig are clearly likely to be less than excreted by the neonate, and it is conceivable that the numbers of mucosalis to which these pigs were exposed were too low to allow establishment of infection in the presence of pharmacologically induced hypomotility. Benzetimide is reported to reduce gastro-intestinal motility in pigs (Marsboom, Temmerman and Symoens, 1973) and although the rate of flow of ingesta was not measured in this experiment, it is assumed that the drug exerted its effect on peristalsis. The possibility that benzetimide has other effects which favoured the establishment of mucosalis cannot be excluded. Benzetimide is an anti-cholinergic agent and may also have influenced gastro-intestinal secretion; gastric secretion is reported as a non-specific factor in resistance to bacterial infection via the oral route (Grady and Keusch, 1971) and an antibody-independent bactericidal mechanism on the mucosal surface of the small intestine related to the intestinal secretions has been described (Klop and Rowley, 1975a and 1975c). In these experiments the gastric contents were neutralised by oral administration of chalk suspension prior to oral dosing with mucosalis.

The recovery of mucosalis from the oral cavity was only achieved once, in this series of experiments. This is in contrast to the isolation rate in neonatal piglets, in which for a number of weeks post oral dosing mucosalis can be isolated from the mouths of at least some members of the litter (chapters IV and VI). This is consistent with an effect of host age on the establishment of oral infection with microorganisms as discussed in chapter VI.

The immunofluorescent and silver staining techniques were not found to be of value in these experiments, where if mucosalis was present, it was probably only present in low numbers. They did however indicate that mucosalis was not present in the tissue in large numbers and yet had not been recovered. The immunofluorescent and bacteriological findings were consistent with the absence of gross or histological changes.

In the pigs in section A the very small amount of fluorescence seen in occasional cells in a few glands, and also in cells in the lamina propria and the mesenteric lymph nodes may have been due to the presence of mucosalis antigen in the epithelium and in macrophages in the lamina propria or lymph node. It is not possible to say whether that fluorescence present in the epithelium represented viable mucosalis in numbers too low to be detected by the cultural techniques used.

In the experiments in section B not all six pigs in each group were dosed orally with mucosalis but they were housed together for the period of the experiment, so it is not impossible that those pigs not dosed, ingested mucosalis, viable or dead, from their pen-mates. Alternatively this fluorescence may be due to fluorescence with a shared antigen. However any fluorescence was of such a low order that one could not definitely affirm that it was due to specific fluorescence of mucosalis antigen. The fluorescence in the cells in the lamina propria may again have been degraded mucosalis antigen in macrophages. It is also possible that other organisms if degraded may share antigens, at that time, with mucosalis.

In those cases in which mucosalis was isolated from the intestinal mucosa, the numbers recovered were low and there was no evidence histologically of adenomatous change in the epithelium. These pigs had

been killed approximately fourteen days post-dosing and it is possible that if they had been killed at a greater time interval after infection lesions may have developed. From the results reported in chapter VII the incubation period for PIA appears to be greatly in excess of fourteen days.

The technique of filtering the mucosa and also the use of inhibitory media (N.B.G.) were found to be useful in reducing the contaminating organisms present in samples. These methods are also likely to quantitatively reduce the numbers of mucosalis present in the sample, but allow the isolation of mucosalis under circumstances when it is probable that it would be overgrown by other members of the gastro-intestinal flora.

The histological changes of villous atrophy and crypt hyperplasia were present in those pigs from which mucosalis was isolated, but were also seen in the other pigs in the study. It is unlikely therefore that they were related to the presence of mucosalis. In the pig these changes have been reported to occur after weaning (Kenworthy, 1971 and 1976) and the proliferation and increased metabolic activity of coliforms within the gut lumen, secondary to the change in diet which occurs at this time, is suggested as the causative factor. The giant cells which were seen in pig 252/74 in the drainage lymph node of the terminal small intestine are of unknown aetiology. They could not be related to the presence of mucosalis, and were only seen in one pig. Giant cells have been reported in PIA by others (Emsbo, 1951; Rahko and Saloniemi, 1972a), however earlier (chapter III) it has been suggested that in the series of pigs studied by the present author giant cells were not seen but that clumps of degenerating epithelial cells resembling giant cells were present. In pig 252/74 there did however definitely appear to be giant cells of both

the Langhan's and the foreign-body type. No explanation for their presence can be offered.

From the results of this series of experiments and those in chapter IV it would seem that post-weaned pigs have a resistance to infection with mucosalis, after oral dosing, in contrast to the neonatal pig. The susceptibility of the post-weaned pig can be increased by pharmacologically induced hypomotility suggesting that gastro-intestinal peristalsis is one of the mechanisms preventing establishment of mucosalis in the post-weaned pig.

This reduction in susceptibility with age has been described for other infectious agents and conditions. In peroral infection with Group B coxsackie virus in adult mice the protective function of the gut has been considered to operate in two ways; a barrier effect which prevents virus passing through the mucosa, and a clearance mechanism which operates to eliminate virus from the intestinal mucosa after infection has occurred (Loria, Kibrich and Broitman, 1974). These protective effects are demonstrable from fourteen to eighteen days of age (Loria, et al. 1976), i.e. from the time of weaning. It may be that this is related to increased cell turnover at that time leading to more rapid loss of virus infected epithelial cells. The cell age is reduced, and the presence of the apical endocytic complex is a function of cell age (Moon, Kohler and Whipp, 1973) which also therefore affects virus uptake. TGE of pigs is a much more severe disease in young piglets than in older animals, and virus production is greater in the neonate than in the older animal. This innate age dependant resistance seems to operate in two ways (Moon, et al. 1975); the increased proliferative capacity of the crypt epithelium from three weeks of age allows a more rapid regeneration of

atrophic villi, and also in the comparatively young villous absorptive cells of the older pig virus production is less than in the older cells of the neonate, since with TGE virus, virus production depends on cell age (Moon, et al. 1975). Whether or not similar factors operate in PIA is unknown. However, since mucosalis occurs intracellularly, it is tempting to suggest that they may play at least some part in explaining the difference in susceptibility of the neonatal pig compared to the post-weaned pig, after oral dosing with mucosalis.

In light of the fact that reduction in peristalsis appears to increase the susceptibility of post-weaned pigs to mucosalis, it is interesting that in suckling piglets the rate of passage of food is slower than in the weaned piglet (Kidder and Manners, 1974).

In experiments with V. cholerae, using adult and neonatal mice (Knop and Rowley, 1975b), it has been shown that there were two differences which could account for the susceptibility of neonatal mice, and the resistance of adult mice to infection (Chaicumpa and Rowley, 1972). Firstly the population of viable V. cholerae is not reduced when passing through the stomach in baby mice, which contrasts greatly with the situation in adult mice. In addition the killing of viable organisms, which reach the small intestine, is much less in baby mice. Knop and Rowley (1975a) also showed the importance of peristalsis in the rapid removal of V. cholerae from the intestines in adult mice.

Although an in vitro effect of virginiamycin on mucosalis could not be demonstrated the possibility cannot be excluded that it exerted an influence in vivo on the orally administered organisms, in part A.

In summary these experiments have shown that the post-weaned pig is relatively resistant to oral challenge with mucosalis under the

circumstances of these experiments. Intestinal infection with mucosalis was not established in conventional animals. Some progress towards elucidating the reasons for this resistance has been made and if pigs also receive chalk, to neutralise the gastric contents, and benzetimide, to reduce gastro-intestinal peristalsis, then intestinal infection with mucosalis can be established. This was demonstrated for a period of approximately fourteen days, and it remains to be determined if PIA will develop later in infections established in this manner.

CHAPTER VI

INFECTIVITY EXPERIMENTS USING NEONATAL PIGLETS,
WITH OBSERVATIONS ON INFECTION OF THE ORAL CAVITY

INTRODUCTION

The type species of the catalase negative Campylobacters, Campylobacter sputorum ss. sputorum is present in the human mouth (Loesche, Gibbons and Socransky, 1965). In man most oral motile organisms, such as spirochaetes and vibrios, are found in highest concentrations in the gingival crevice area (Gibbons and Van Houte, 1975). Little or no saliva penetrates into the gingival crevice and it is thought that when such motile organisms are introduced into the mouth, active movement enables them to reach this quiescent site (Gibbons and Van Houte, 1975). Human oral microbiology has been a field of active research investigating the involvement of bacteria in a number of dental diseases. There has been little reported veterinary work in this area.

Campylobacter sputorum ss. mucosalis has been isolated from the mouths of piglets (Lawson, Rowland and Roberts, 1975). This was a limited study but provided information on the presence of mucosalis in, and the isolation of mucosalis from, the oral cavity of animals without gross or clinical evidence of PIA. In the experiments described in Chapter IV mucosalis was isolated from the mouths of piglets at varying intervals post-dosing. Similarly, it has also been isolated from the mouth in cases of PIA (Chapter III) (Lawson, Rowland and Roberts, 1976). The catalase negative Campylobacters of the porcine oral cavity seem to be a

heterogenous group and some isolates are biochemically and serologically typical of mucosalis (1248/72 2C2), others are biochemically typical but serologically distinct, while a third group are both biochemically and serologically different from mucosalis (Lawson, Rowland and Roberts, 1976).

In this experiment piglets were orally dosed with mucosalis in the neonatal period and maintained with the sow. The piglets were monitored clinically and weekly oral swabs were examined bacteriologically from each of the piglets for the presence of *Campylobacters*. Particular emphasis, in swabbing, was paid to the gingival margin.

MATERIALS AND METHODS

Experimental Animals and Timing of Procedures

A Duroc sow from a local breeding research organisation (farm A) was introduced to the isolation block several days before farrowing. She farrowed nine live piglets. These were removed from the sow before suckling and eight were dosed orally with mucosalis approximately 90 minutes after the birth of the last piglet. The piglets were returned to the sow 1.5 hours later, i.e., 2.5 hours after birth of the last piglet. All eight piglets received oral chalk suspension before the mucosalis suspension.

One of the piglets which had received the diluted mucosalis inoculum was killed at 24 hours of age. The uninoculated control piglet was killed at 37 days of age.

The piglets were weaned at 51 days of age. They were offered creep (Starter Creep - Seafield Mill) from 10 days of age and ABRO Breeders

meal from three weeks of age.

Mouth swabs were taken from all of the piglets at two days of age and weekly from then onwards until 72 days of age. Mouth and vaginal swabs were taken from the sow on days 30, 37, 44 and 51 of the experiment.

The remaining seven piglets were killed at 80 days of age and subjected to post-mortem examination. Bacteriological examination was not carried out on these piglets at this time.

Sampling

A sterile cotton wool swab was rotated along the gingival margin of the piglet. In the laboratory the swab was broken off into a McCartney bottle containing 5 ml sterile saline and shaken for five minutes. Each sample was then treated as described in Chapter II.

Infecting Inoculum

The suspension of mucosalis used to orally dose four of the piglets contained 3.5×10^8 viable mucosalis per ml. The other four orally dosed piglets received a ten-fold dilution in tryptose phosphate broth of that suspension, i.e. containing 3.5×10^7 viable mucosalis per ml. Each dosed piglet received 15 mls of one of the mucosalis suspensions. The cultures were prepared as described in Chapter II using strain 106/75 SI 10^{-3} .

RESULTS

Daily Observations/Clinical Findings

Twenty four hours post-oral inoculation: one of the piglets which had received the ten-fold diluted mucosalis suspension was weak and

not suckling and was therefore killed. Up to six weeks of age the remaining piglets showed a marked variation in size and in the amount of creep taken.

From 21 days of age the uninoculated control piglet was noticeably smaller in size and less active compared to its litter mates. Little creep was taken by this piglet but some meal was taken if mixed with water to give it a very loose consistency. This piglet was killed on day 37 as it had become progressively weaker although on no occasion was diarrhoea seen. From approximately six weeks of age the other piglets began to take more food and improve in bodily condition.

Pathological Results: Gross Findings

142/75

At post-mortem the orally dosed piglet which was killed at 24 hours of age, failed to reveal any abnormalities. The stomach contained some milk, the small intestine was almost empty and there was some meconium in the large intestine. 30 cms of the ileum, 30 cms proximal to the ileo-caeco-colic valve was thought to be thickened. Examination under a low-power dissecting microscope showed this to be due to clumping of the villi.

184/75

The uninoculated control piglet killed at 37 days of age was in very poor bodily condition. No abnormalities were detected in the thoracic and abdominal viscera except in the alimentary tract. The stomach was full of clotted milk, and the small intestine was dilated and contained a yellow fluid of mucus consistency with flecks of clotted milk. Some

loops of the small intestine were gas-filled. Few villi could be seen on the surface of the small intestine which had a gelatinous appearance. Peyer's patches were prominent in the lower ileum. The large intestine contained a green/yellow sticky fluid, again with flecks of clotted milk. Most of the mucosa of the large intestine appeared thickened and nodular. The ileo-caeco-colic lymph nodes were firm, grey/white and cellular, with a follicular appearance on the surface and cut surface.

The remaining 7 piglets in the litter

At 80 days of age when the remaining seven piglets were necropsied no gross lesions were seen in the alimentary tract or the carcasses.

Histopathology

Piglet killed at 24 hours post-dosing (142/75)

No abnormalities were noted in the intestine of this piglet. In the mid-small intestine the villi were long and finger-shaped, in the epithelial cells the nuclei were apical in position. There was no reaction in the lamina propria. In the terminal small intestine the epithelial cell nuclei were basal in position; Peyer's patches were visible but only poorly developed. The structure of the large intestine was unremarkable.

Control (uninoculated) piglet killed at 37 days of age (184/75)

Mid-small intestine/terminal small intestine

There was a severe villous atrophy and crypt hyperplasia and a high mitotic index. The epithelial cells of the glands had a basophilic cytoplasm and varied in shape from cuboidal to columnar, with some even more flattened. There were mucus-secreting cells present, but these were not a prominent feature. The lamina propria was unremarkable.

Large intestine

There was a considerable variation in gland size and shape. The epithelial cells varied from cuboidal to flattened, and occasional goblet cells only were present (Figure 58). The impression gained was one of exhaustion of the epithelium and in P.A.S. stained sections the epithelial cells did not stain for mucus, although mucus was present in the lumen of the glands. In some areas the surface epithelium was damaged and associated with this there were neutrophils in the lamina propria. In the lamina propria there was an increased number of cells; lymphocytes, macrophages and plasma cells. In a number of glands there were degenerating neutrophils present and in some cases also within the epithelium.

Bacteriological Results

Bacteriological Findings in Piglets (Post-Mortemed) at Days 1 and 37

Pig 142/75 (24 hours post-inoculation)

The terminal small intestine, only, of this piglet was examined bacteriologically and mucosalis was not isolated. No other significant bacteriological findings were made.

Pig 184/75

Immediately after euthanasia the mouth, and mid-small intestine, terminal small intestine and large intestine of this piglet were examined bacteriologically and mucosalis was not isolated from any of these sites. C. coli was isolated from the mouth and all three sites in the intestine of this piglet.

Bacteriological Results of Examination of Mouth Swabs

Starting at two days of age, i.e. 2 days post-oral dosing with mucosalis, a mouth swab from each piglet was examined weekly. The presence

of Campylobacters was noted. The results are summarised in Table 6a.

In this table the piglets are as follows:

Control - was not orally dosed with mucosalis.

Piglets N1, N2, N3 and N4 - received the undiluted suspension of mucosalis.

Piglets D1, D2 and D3 - received a 1/10 dilution of the mucosalis suspension received by piglets N1, N2, N3 and N4.

The Campylobacters (Vibrios) are as follows:

Mucosalis and C. coli are as described in Chapter II.

Catalase negative, slide agglutination negative Campylobacters are those Campylobacters which are catalase negative in slide catalase tests and do not react in slide agglutination tests with mucosalis anti-serum (1248/72 2C2 OH). These isolates were not subjected to extensive biochemical investigation, although a number of mouth isolates have been examined (Lawson, Rowland and Roberts, 1976) and found to be characteristic of the genus Campylobacter. These isolates are therefore referred to as Campylobacters although the possibility that some of them may not belong to this genus cannot be definitely excluded. In the table they are referred to as non-agglutinating Campylobacters (N.A.C.s).

The recovery of mucosalis from the mouths of these piglets is summarised in Table 6b. With the exception of week 2 mucosalis was isolated from a variable number of piglets each week, up to 8 weeks post-infection. The number of piglets from which mucosalis was isolated increased to a maximum at week 5, and then decreased until no recoveries were made in weeks 9, 10 and 11. Mucosalis was not isolated from the same piglets each week, except for piglet D3 which proved to be infected in 6 of the 7 weeks in which it was isolated from some members of the litter.

Table 6a Results of the Examination of Mouth Swabs for Campylobacters.

Pigs		N1	N2	N3	N4	D1	D2	D3
Control								
Week 1 2 days old	<u>C. coli</u>	<u>Mucosalis</u>	N.R.	N.R.	N.R.	N.R.	N.R.	<u>Mucosalis</u>
Week 2 9 days old	N.R.	<u>C. coli</u>	<u>C. coli</u>	N.R.	N.R.	N.R.	N.R.	N.R.
Week 3 16 days old	<u>C. coli</u>	N.R.	N.R.	<u>Mucosalis</u>	N.A.C.s	<u>C. coli</u>	<u>Mucosalis</u>	<u>Mucosalis</u>
Week 4 23 days old	<u>Mucosalis</u>	N.R.	<u>C. coli</u>	<u>C. coli</u>	N.R.	<u>Mucosalis</u> <u>C. coli</u>	N.R.	<u>Mucosalis</u>
Week 5 30 days old	<u>Mucosalis</u>	<u>Mucosalis</u>	<u>Mucosalis</u>	<u>C. coli</u>	<u>C. coli</u>	<u>Mucosalis</u>	<u>Mucosalis</u>	<u>Mucosalis</u>
Week 6 37 days old	Euthanasia <u>C. coli</u> mouth and intestines	<u>C. coli</u>	<u>C. coli</u> N.A.C.s	N.R.	<u>Mucosalis</u>	<u>C. coli</u> <u>Mucosalis</u>	<u>C. coli</u>	<u>Mucosalis</u>

Table 6a (Cont'd)

Pigs		N1	N2	N3	N4	D1	D2	D3
Control								
Week 7	N.D.	<u>C. coli</u>	<u>C. coli</u>	<u>C. coli</u>	N.R.	<u>C. coli</u>	<u>C. coli</u>	<u>Mucosalis</u> <u>N.A.C.s</u>
45 days old								
Week 8	N.D.	N.R.	<u>C. coli</u> <u>N.A.C.s</u>	<u>C. coli</u>	<u>Mucosalis</u> <u>C. coli</u>	<u>C. coli</u> <u>N.A.C.s</u>	<u>C. coli</u>	<u>N.A.C.s</u>
51 days old								
Week 9	N.D.	<u>C. coli</u> <u>N.A.C.s</u>	<u>C. coli</u>	<u>C. coli</u> <u>N.A.C.s</u>	<u>C. coli</u> <u>N.A.C.s</u>	<u>C. coli</u> <u>N.A.C.s</u>	N.R.	N.R.
57 days old								
Week 10	N.D.	N.R.	N.R.	N.R.	N.R.	N.R.	<u>C. coli</u>	N.R.
64 days old								
Week 11	N.D.	<u>N.A.C.s</u>	<u>C. coli</u>	<u>C. coli</u>	N.R.	N.R.	<u>C. coli</u>	<u>C. coli</u>
72 days old								

N.D. = Not done

N.R. = No campylobacters recovered

Table 6b The Recovery of Mucosalis and N.A.C.s

Week	Numbers of Piglets from which <u>Mucosalis</u> isolated	Piglets from which <u>Mucosalis</u> was isolated	Numbers of piglets from which N.A.C.s isolated
1	2/8	N1, D3	0/8
2	0/8		0/8
3	3/8	N3, D2, D3	1/8
4	3/8	Control, D1, D3	0/8
5	6/8	Control, N1, N2, D1, D2, and D3	0/8
6	3/8	N4, D1, D3	1/8
7	1/7	D3	1/7
8	1/7	N4	3/7
9	0/7		4/7
10	0/7		0/7
11	0/7		1/7

With the exception of the single isolation of catalase negative, slide agglutination negative campylobacters made from piglet N4 in week 3, catalase negative, slide agglutination negative campylobacters were not made until week 6, after which time they were isolated from a greater proportion of the piglets (see Table 6b).

Piglet D3 is of interest since mucosalis was isolated from the oral cavity of this piglet each week, except for week 2, up to week 7. In week 7 both mucosalis and catalase negative, slide agglutination negative campylobacters were isolated from the same swab. In week 8 only catalase negative, slide agglutination negative campylobacters were isolated from the oral cavity of this piglet.

The results of bacteriological examination of mouth and vaginal swabs from the sow are summarised in Table 6c. Mucosalis was not isolated from either the oral cavity or vagina on any occasion. Catalase positive campylobacters were isolated from both sites and on one occasion catalase negative, slide agglutination negative campylobacters were also recovered from the mouth.

In summary, mucosalis could be isolated for up to eight weeks from a number of the piglets in this litter after oral dosing, by the methods employed in this study. It was not isolated from the same pigs each week, although some pigs were positive for a number of consecutive weeks, mucosalis was recovered from the mouth of one pig on 6 out of 7 weeks. It would seem that mucosalis can persist in the oral cavity of litter pigs for up to 8 weeks after oral dosing. Spread also took place from orally dosed piglets to an undosed litter mate, maintained with them. Mucosalis was not isolated from the oral cavity of the sow.

Table 6c Results of Bacteriological Examination of Mouth and Vaginal Swabs from the Sow

Days Post-infection of the Litter	Mouth	Vagina
30	No campylobacters isolated	No campylobacters isolated
37	<u>Mucosalis</u> not recovered, but other catalase negative slide agglutination negative campylobacters and catalase positive campylobacters isolated.	<u>Mucosalis</u> not recovered, but catalase positive campylobacters isolated.
44	<u>Mucosalis</u> not recovered, but catalase positive campylobacters isolated.	<u>Mucosalis</u> not recovered, but catalase positive campylobacters isolated.
51	No campylobacters isolated	No campylobacters isolated

Immunofluorescence Results

Only tissue from pig 184/75 was examined by immunofluorescence. All sites, mid-small intestine, terminal small intestine, and large intestine, were negative for particulate fluorescence when stained with 1248/72 2C2 mucosalis anti-sera and F.I.T.C. conjugated G.A.R. serum.

E.M. Findings

184/75

A limited amount of tissue from the large intestine of pig 184/75 was examined, no intracellular bacteria or significant ultrastructural changes were seen in the grids examined.

DISCUSSION

The establishment of oral infection with mucosalis in a litter of neonatal piglets after oral exposure is described. The infection was demonstrable irregularly for up to eight weeks in this litter of pigs. The method of sampling was relatively crude and it cannot necessarily be said that mucosalis was absent from the mouths of those piglets from whose mouth swabs it was not isolated. It seems reasonable, however, that, taken as a whole, these results provide an indication of the presence of mucosalis within the oral cavity of some members of the litter.

The duration of infection of eight weeks is comparable to the period of clinical symptoms in PIA (Rowland and Rowntree, 1972), the experimental disease (Roberts, Rowland and Lawson, 1977; Chapter VII),

and the persistence of infection in the intestinal tract of orally dosed neonatal piglets (Chapter IV). There was spread from dosed piglets to the undosed piglet. With Streptococcus mutans in rats transmission from mouth to mouth occurs mainly by coprophagy (Van Houte et al., 1976). These workers suggest that the faecal count of S. mutans in their experiments was directly related to the mouth population of the organism. It is possible that spread by coprophagy occurred within this litter of piglets. Mucosalis in faeces could also have been derived from any intestinal infection which may have established. The faeces were not examined bacteriologically and bacteriological examination of the intestine was only carried out on two piglets from this litter. In view of the results reported in Chapter IV it is likely that intestinal infection did establish in at least some members of the litter. Spread could also have occurred by suckling contaminated teats.

During the period of the experiment, there was no evidence of PIA although a number of piglets grew only relatively slowly, they were healthy and never thin. In pig 184/75 at necropsy there were no gross lesions of PIA and histologically there was no frank adenomatous change. The histological picture resembled in a number of features the three week-scouring problem described in Chapter III. When the piglets were necropsied at the termination of the experiment, i.e. 11 weeks after oral dosing with mucosalis, there were no gross changes of PIA visible. Even if present in this experiment one would have expected them to have resolved by this time (Chapter VII).

In man, bacteria colonise the mouths of infants shortly after birth. It is believed that most indigenous oral micro-organisms

are acquired from the parents or attendants of the infants, as few of these bacteria have been reported to be free-living in nature (Gibbons and Van Houte, 1975). In experiments on monkeys using Lactobacillus salivarius Bowen (1968) showed that transfer of the organism from mother to the offsprings' mouths occurred more readily than between mature animals. To date mucosalis has not been isolated from the oral cavity of adult pigs and the significance of this site as a focus of possible infection remains unknown as only a very small number of adult animals have been examined in this manner (Lawson, Rowland and Roberts, 1975). A sow orally infected could provide a source of the organism for her litter. In these experiments and those described in Chapter IV the infected animals did not develop overt signs of disease, also the natural disease is not seen in this age of pig. If, however, infection did occur in neonatal animals they may act as a source of infection for other pigs at weaning when the circumstances for establishment of infection and development of disease might differ.

Little information is available about the establishment of micro-organisms in the oral cavity (Van Houte, 1976) although the host's age is becoming recognised as important in oral colonisation by bacteria. In rats younger animals are more susceptible to infection with S. mutans (Van Houte and Upeslacis, 1976) whereas in the same type of rat susceptibility to oral infection with Actinomyces viscosus increases after weaning (Brecher and Van Houte, 1976). It is interesting that a number of isolations of mucosalis were made from the mouths of orally dosed neonatal piglets but only one isolation from the post-weaned group (Chapters IV and V). The

susceptibility of the neonatal piglet to oral infection with mucosalis is confirmed in this chapter.

In a manner analogous to the rapid clearance of bacteria from the intestine, Bloomfield (1919, 1920a, b) demonstrated that various bacteria when introduced into the mouth and nasopharynx were rapidly cleared. There is a great variability in the bacterial composition of the oral cavity in the first few days of life (Burnett, Scherp and Schuster, 1976). Possibly during this period, establishment of introduced organisms is easier. The motility of mucosalis may allow it to attain the gingival crevice, where the flow of crevicular fluid is less than that of the saliva bathing the rest of the mouth. Conditions in the gingival crevice are highly reduced (low oxygen tension) and also rich in nutrients supplied by the crevicular fluid (Gibbons and Van Houte, 1975). In the intestine, mucosalis is present in an intracellular site. Its location in the oral cavity is unknown.

In this experiment in addition to the recovery of mucosalis from the oral cavity, other catalase negative, serologically different Campylobacters were isolated. The indication was that these appeared and certainly increased in number after mucosalis started to disappear. Further work is necessary to repeat this and also to examine the other Campylobacters recovered. Antigenic variation has been described in Streptococcus mutans colonising the intestine of gnotobiotic rats (Bratthall and Gibbons, 1975b) and the suggestion has been made that this variation is due to the selection pressure by antibodies to the bacteria. The evidence

presented for this is the changing agglutinating activities of salivary immunoglobulin A against oral streptococci over a period of time (Bratthall and Gibbons, 1975a). It remains to be established if the other catalase negative Campylobacters are the result of such selection pressure mediated by antibodies against mucosalis. It may be that these other Campylobacters were present already and on the disappearance of mucosalis they are able to increase in number in the same ecological niche previously dominated by mucosalis. Alternatively these could have been derived from the sow, catalase negative Campylobacters other than serologically typical mucosalis (1248/72 2C2) have been isolated from this sow and also other sows (Chapter IV). In sow D (Chapter IV), catalase negative campylobacters, ~~which did not react in~~ slide agglutination tests with 1248/72 2C2 mucosalis inti-sera (N.A.C.s), were recovered from the oral cavity six days before farrowing. At six days after oral dosing with mucosalis, two out of four piglets from her litter were positive for N.A.C.s, and mucosalis was not recovered from any of the four. At twelve and twenty five days post exposure to mucosalis, mucosalis was recovered from two out of three, and three out of four piglets sampled. It is tempting to suggest that the piglets acquired the N.A.C.s from the sow. As already discussed, mucosalis in the mouth was probably acquired through coprophagy. Once present in the piglets of the litter of sow D, mucosalis seemed to replace the other Campylobacters.

At this time any comment is only conjectural, and further clarification depends on a closer examination of the Campylobacters in the porcine oral cavity. It is probably relevant that the

situation in man also appears complex.

In man, C. sputorum ~~ss~~ sputorum has been recovered from the mouth (Loesche et al., 1965). Van Palenstein Helderma and Rosman (1976) described vibrios in the oral cavity which biochemically resembled Campylobacter sputorum but had an absolute hydrogen dependence and a GC ratio of between 48% and 50%, prompting the authors to suggest that they should revert to their original name of Vibrio sputorum as opposed to Campylobacter sputorum. Although this taxonomic suggestion bears little attraction to the present author it is clear that mucosalis is closely related to some of the oral vibrios of man. More recently, Crawford et al., (1976) have examined the biochemical characteristics and D.N.A. base composition of oral vibrios and recognise two groups. A microaerophilic group which exhibited characteristics consistent with the genus Campylobacter and having a moles % G. & C. of 33 - 36. A strictly anaerobic group had similar biochemical characteristics but a moles % G. & C. of 42-47.

As well as antibodies salivary glycoproteins are thought to prevent attachment of oral micro-organisms (Williams and Gibbons, 1975). More recently it has been shown that S. mutans and probably other oral bacteria can bind blood group reactive substances from human saliva. In this way they acquire the blood group reactivity of their host while proliferating in the mouth (Gibbons and Qureshi, 1976). It would be of interest to determine if mucosalis has this ability in view of the lack of response to mucosalis in its intracellular situation in the intestinal epithelium (Rowland and Lawson, 1974).

In conclusion, this experiment has demonstrated that after oral exposure mucosalis can become established and be maintained in the oral cavity of a litter of piglets for up to 8 weeks. Although the true significance of this is at present unknown, a number of interesting facets of the relationship are discussed.

SECTION B

(CHAPTERS VII AND VIII)

PORCINE INTESTINAL ADENOCARCINOMA

- TRANSMISSION EXPERIMENTS

INTRODUCTION TO THE TRANSMISSION EXPERIMENTS

In the introduction to the infectivity experiments the problems of, and some of the factors involved in, the establishment of enteric infections with particular organisms were discussed. The author would now like to consider the experimental reproduction of disease, discussing in particular the porcine proliferative enteropathies and those conditions in other species with features in common with PIA, namely proliferative ileitis in the hamster, and Tyzzer's disease associated with Bacillus piliformis.

Over the years many attempts have been made to reproduce experimentally PIA or RI. To date no successful reproduction has been documented. As early as 1929 - 1931 Adersen (cited by Emsbo, 1951), attempted unsuccessfully to transmit the condition using bacterial cultures from the intestine, minced organ material and contact. Biester and Schwarte (1931) fed intestinal contents and scrapings from the large intestine of affected pigs to twelve pigs, which developed an acute dysentery. In four of these animals examined histologically, an epithelial proliferative process in the caecum and colon was described. This is not illustrated and due to the lack of knowledge on swine dysentery at that time and the presence of intercurrent infection with Salmonella suispestifer it is difficult to evaluate their results. In 1939 Biester, Schwarte and Eveleth carried out further transmission studies using tissue from an affected pig. There was no other infective condition involved in this instance, and transmission was unsuccessful. Others have also attempted without success to reproduce the condition using material

from affected animals (Emsbo, 1951; Korpassy and Tiboldi, 1957; Rowland and Rowntree, 1972; and others).

Hoorens (1962) carried out a number of experiments involving the feeding of talc and silica gel, surgical procedures including blockage of the lymphatics to the gut, partial occlusion of the gut lumen and injection of sclerosing substances into the gut wall and lymphatics. The changes produced were those to be expected from such surgical interference and introduction of irritant foreign substances; oedema, connective tissue proliferation and a foreign body response. From his work Hoorens concluded that although the primary cause of RI was still unknown, obstruction of the lymphatics of the gut wall and mesentery were important in the pathogenesis.

Workers in Finland have also suggested that lymphatic obstruction may play an important role in the pathogenesis of RI (Kalima, 1971) and have used this hypothesis for the basis for their experimental work. Granulomatous inflammation with foreign-body giant cells in the gut wall was produced after experimental lymphatic obstruction using sclerosing substances (Rahko, Saloniemi and Kalima, 1973; Saloniemi, Rahko and Kalima, 1974; Kalima, Saloniemi and Rahko, 1976). This transmural inflammation with round cell aggregations (Kalima et al., 1976), in which epithelial hyperplasia was not described clearly differs from PIA and RI.

Tyzzar's disease is a condition first reported in 1917 in mice by Tyzzar. Since that time it has been recorded in the mouse, rat, hamster, gerbil, rabbit, cat, sub-human primates (Ganaway, Allen and Moore, 1971), the dog (Qureshi, Carlton and Olander, 1976), and

horses (Swerczeck, et al., 1973). It is caused by an intracellular bacterium, Bacillus piliformis which, like Mucosalis lies free within the cytoplasm of host cells but differs in ultimately causing necrosis. Great difficulty has been experienced in isolating B. piliformis and up to 1966 the only sources available for experimental transmission were infected mouse liver and brain (Craigie, 1966a). Kanazawa and Imai (1959) claimed to have isolated B. piliformis on artificial media, although some of the characters of their organism suggest that it was possibly not B. piliformis (Ganaway, Allen and Moore, 1971). More recently isolation on artificial media has been described from rabbits by Simon (1977). Rights, Jackson and Smadel (1947) thought that they had isolated B. piliformis in mouse embryo cell cultures, but virulence for mice was lost after the first passage. They concluded that B. piliformis rapidly lost virulence in culture, or a symbiote necessary for production of disease failed to grow in their cultures. B. piliformis has been isolated using embryonated hen's eggs and used as a source of infective material to produce the disease (Craigie, 1966a, 1966b; Ganaway, Allen and Moore, 1971). The vegetative form of B. piliformis rapidly loses its infectivity in vivo, the organism undergoing lysis once the animal dies (Ganaway, Allen and Moore, 1971). Also in ovo, the vegetative phase undergoes rapid lysis after the embryo dies, so that residual infectivity in such cases is probably due to the presence of spores (Craigie, 1966a, 1966b). Craigie found that in yolk-sac suspensions there was a marked loss of infectivity within fifteen to twenty minutes at room temperature, the loss being more rapid at 37°C and infectivity being completely lost after twenty four hours at 4°C. Fujiwara et al., (1963 and 1965) noted a

marked loss of the bacillus in suspensions of infected mouse liver, stored in vitro. The vegetative phase is destroyed by freezing and storage at low temperatures (-70°C) (Fujiwara et al., 1965; Ganaway, Allen and Moore, 1971). Craigie, working with a non-sporing variant, harvested the yolk sac of eggs before the embryo died, and froze them within fifteen minutes of harvest. After this procedure and storage at -75°C there was a loss of more than 99% of the original infectivity.

The route of infection in Tyzzer's disease is most probably oral (Ganaway, Allen and Moore, 1971; Swerczek, 1977) and spread from the alimentary tract, where changes may or may not be detected, results in liver and myocardial lesions. Reproduction of Tyzzer's disease by oral administration of affected material has produced varying results (Ganaway, Allen and Moore, 1971), which is perhaps not surprising considering the fragility of the organism. Until the usefulness of cortisone treatment in this work was recognised, transmission experiments, including the intravenous injection of affected material, did not always result in disease (Craigie, 1966a, 1966b; Ganaway, Allen and Moore, 1971).

In summary then, Tyzzer's disease can be transmitted from animal to animal, but in handling affected tissue the lability of the organism has to be remembered. Cortisone treatment enhances the reproduction of disease.

Proliferative ileitis in the hamster is a condition with many features in common with PIA; clinically, epidemiologically and with respect to gross pathology and histopathology the two conditions

are very similar. Free within the apical cytoplasm of affected intestinal epithelial cells there are bacterial forms (Wagner, Owens and Troutt, 1973) as in PIA. Wagner et al. (1973) recovered E. coli 0138 closely related to Shigella boydii types 11 and 12, from the affected tissues in cases of proliferative ileitis, but not from normal hamsters. However, they were unable to reproduce the condition using cultures of this organism. It has not been conclusively demonstrated that the intracellular organism is E. coli 0138 resembling S. boydii types 11 and 12. Experimental transmission of proliferative ileitis in the hamster has been reported (Jacoby, Osbaldiston and Jonas, 1975; Amend et al., 1976; Frisk, Wagner and Owens, 1977). Jacoby et al. (1975) were able to reproduce the condition by feeding segments of ileal lesions, homogenates of ileal lesions, whole-cell free supernatants of ileal homogenates and with histologically normal segments from otherwise diseased intestine. Homogenates heated to 56°C for thirty minutes, exposed to chloroform or passed through bacteria-retaining filters did not transmit the disease.. Mixed bacterial flora isolated from ileal lesions capable of inducing disease, also induced disease but with a lower morbidity and mortality. Pure cultures of organisms isolated from the mixed flora did not produce the condition, with one exception. A slow lactose fermenting E. coli in one experiment apparently induced proliferative ileitis, but the authors considered the result to be of doubtful significance. Amend et al. (1976) and Frisk et al. (1977) were also able to reproduce proliferative ileitis by feeding homogenised affected tissue to susceptible animals. A

slow lactose fermenting E. coli was recovered, by the first named authors, consistently from the lesions, but cultures of this organism, alone or with lactose fermenting E. coli or with bacteria-free filtrates, failed to induce proliferative ileitis.

These three conditions have a number of features in common. All are associated with intracellular bacteria, which lie free within the host cell cytoplasm. The recovery of bacteria from these conditions has proved difficult, although for PIA it has been achieved, and convincingly demonstrated that Campylobacter sputorum subsp. mucosalis is the intracellular organism. This bacterium is recovered consistently from PIA cases, and has been characterised (Lawson, Rowland and Wooding, 1975; Lawson, Rowland and Roberts, 1976).

Using the information obtained from the infectivity experiments with mucosalis (Chapters IV, V and VI) and taking regard of the work discussed here, experiments were undertaken to reproduce PIA.

CHAPTER VII

TRANSMISSION EXPERIMENTS USING NEONATAL PIGLETSINTRODUCTION

In the infectivity experiments (Chapters IV, V and VI) the susceptibility of the neonatal piglet to infection with mucosalis was found to be greater than that of the older, post-weaned pig. Whether dosing was carried out before or after suckling and intake of colostrum, did not seem to affect the susceptibility to mucosalis infection, but the site from which mucosalis was recovered, varied. Benzetimide induced hypomotility was also shown to be important, in the post-weaned pig, in establishing infection. In the one litter of neonatal piglets in which there was some histological evidence of PIA, the piglets had received benzetimide. Neonatal piglets were therefore used in this series of experiments and these piglets received benzetimide to reduce gastro-intestinal peristalsis.

Fresh mucosa was obtained from confirmed cases of PIA if available. This was used to prepare a homogenised suspension immediately after euthanasia of the donor animal. On occasion it was found impossible to locate suitable diseased donor animals coincident with the onset of farrowing, in these instances homogenised mucosa stored at -80°C was employed. Infection of piglets was carried out as soon as possible after the preparation of the material whether fresh or frozen mucosa was used.

MATERIALS AND METHODS

Infecting Organism

The mucosalis strain 106/75 was used in these experiments. The numbers of viable mucosalis received by these piglets, which were orally dosed with mucosalis is listed below:

Litter ex sow F	9.5×10^9
Litter ex sow G	1.0×10^{10}
Litter ex sow H	8.5×10^9

Source of mucosa used for dosing

Piglets in the litter of sow F received mucosa from two pigs: 71/76 (see Chapter II) and 76/76 (see Chapter III). Two pigs 87/76 and 88/76 (see Chapter III) were used to provide fresh mucosa for dosing piglets in the litter of sow G, and after it had been stored at -80°C , to dose piglets in the litter of sow H.

The estimated numbers of viable mucosalis per gram of adenomatous mucosa in donor animals is presented in Chapter III. The suspensions of mucosa for dosing were prepared as described in Chapter II. Each piglet received five mls of homogenised suspension, each time it was dosed with mucosa. The suspensions contained equal volumes of mucosa and tryptose phosphate broth.

Benzetimide

The benzetimide was given orally at a dose of 0.250 mgs per kg body weight. The required volume of a 0.025% solution was made up to approximately ten ccs in sterile distilled water.

Experimental Animals and Timing of Procedures

The piglets used in these experiments were born to three sows obtained from herd A. They were introduced into clean, recently formalin-fumigated pens in the isolation block several days before farrowing. The sows were fed 'A.B.R.O. Sow and Weaners' meal (Seafield Mill, Roslin). These sows will be referred to as F, G and H.

Sow F

Eleven piglets born to sow F were removed at birth, before suckling. All eleven of these piglets received orally one gram of chalk in distilled water, and benzetimide. Six of them were orally dosed with a mucosal suspension prepared from a pig suspected on clinical grounds to be affected with PIA, but which did not histologically show frank adenomatous change and from which mucosalis was not recovered (pig 71/76), and they also received a suspension of mucosalis. The remaining five piglets were dosed only with a suspension of mucosalis. After dosing all eleven piglets were returned to the sow. At twenty four hours old, four of the piglets which had already been dosed orally with both mucosa and a suspension of mucosalis, received orally a further suspension of mucosa from a field case of PIA (pig 76/76). Two piglets which were born after the original eleven had been returned to the sow, were also dosed with this mucosa. These last two piglets received no other treatment, and had suckled before dosing. From thirteen days of age the piglets were offered creep pellets ('Creep Starter' pellets - Seafield Mill, Roslin). The piglets were weaned at twenty seven days of age and

were offered 'A.B.R.O. Sow and Weaners' Meal from that time, and the quantity of creep pellets fed was reduced.

From forty seven days of age the remaining piglets in the litter were mixed with the piglets still alive from the litter of sow G, both groups being moved to a clean, freshly-fumigated pen in the isolation block.

The piglets were weighed at thirteen and twenty seven days of age, and approximately weekly thereafter. Piglets were killed sequentially, selection of the poorest growing pigs being made from the CUSUM values. Table 7a summarises the treatment each piglet received and the age at death or euthanasia.

Sow G

Sow G farrowed eleven piglets overnight. Two piglets were killed at approximately twelve hours of age as "negative" controls. The remaining nine piglets were orally dosed at approximately eighteen hours of age. They received chalk, benzetimide, a mucosal suspension prepared from pigs 87/76 and 88/76, and a suspension of mucosalis. From eight days of age the piglets were offered creep pellets, and they were weaned at twenty two days of age. After weaning the pigs received reducing quantities of creep pellets, and were offered 'A.B.R.O. Sow and Weaners' meal ad-lib. Water was available ad-lib. From forty three days of age the piglets in the litter which were still alive were mixed with the remainder of the litter of sow F.

The piglets were weighed at nine and twenty three days of age, and then at approximately weekly intervals for the duration of the experiment. Pigs were killed sequentially throughout the experiment, selection being based on clinical assessment and the CUSUM

Table 7a Details of Piglets in the Litter of Sow F

Pig Reference	Age at Dosing	Dosed with:	Age at Death or Euthanasia	
74/76	Birth	Benzetimide and <u>Mucosalis</u>	Died	12 hrs
75/76	Birth	Benzetimide, Mucosa (71/76) and <u>Mucosalis</u>	Died	12 hrs
80/76	Birth	Benzetimide and <u>Mucosalis</u>	Died	24 hrs
81/76	Birth	Benzetimide, Mucosa (71/76) and <u>Mucosalis</u>	Euthanasia	24 hrs
108/76	Birth and 24 hrs	Benzetimide, <u>Mucosalis</u> and Mucosa (71/76 and 76/76)	Euthanasia	12 days
133/76	Birth	Benzetimide and <u>Mucosalis</u>	Euthanasia	35 days
134/76	Birth and 24 hrs	Benzetimide, <u>Mucosalis</u> and Mucosa (71/76 and 76/76)	Euthanasia	35 days
141/76	24 hrs	Mucosa (76/76)	Euthanasia	42 days
190/76	24 hrs	Mucosa (76/76)	Euthanasia	64 days
209/76	Birth	Benzetimide and <u>Mucosalis</u>	Euthanasia	70 days
216/76	Birth	Benzetimide and <u>Mucosalis</u>	Euthanasia	76 days
217/76	Birth and 24 hrs	Benzetimide, <u>Mucosalis</u> and Mucosa (71/76 and 76/76)	Euthanasia	76 days
219/76	Birth and 24 hrs	Benzetimide, <u>Mucosalis</u> and Mucosa (71/76 and 76/76)	Euthanasia	77 days

values, derived from the figures for body weights. Pigs were killed at the following times post-dosing: 23 days (1), 37 days (1), 50 days (1), 52 days (1), 58 days (2), 64 days (2) and 65 days (1).

Sow H

Sow H was introduced into a farrowing crate in a pen in the isolation unit several days before farrowing. She was alert and bright but reluctant to stand. She was anorexic before parturition although ~~a~~ febrile, and farrowed ten live piglets and one still-born. The piglets were dosed at twenty four hours of age with chalk, benzetimide, a suspension of mucosalis, and adenomatous mucosa (from pigs 87/76 and 88/76, after storage at -80°C). Eighteen hours after dosing two of the piglets were moribund and two others weak. The sow appeared to have little milk and was given oxytocin by intramuscular injection in an attempt to stimulate milk let-down. The two moribund piglets were killed and necropsies carried out. At thirty six hours post-dosing one of the previously weak piglets was dead. At sixty hours post-dosing, the other piglet, which had been weak at eighteen hours post-dosing, was dead. From that time the piglets were offered milk (S.M.A. - John Wyeth and Brother Ltd., Berks) with creep pellets added. The sow received 'A.B.R.O. Sow and Weaners' meal, and water was always available. The piglets were weighed at thirteen days post-dosing, and approximately weekly thereafter. Weaning was carried out at twenty two days of age, and the piglets were fed 'A.B.R.O. Sow and Weaners' meal, and water was always available. The remaining six piglets were killed at 34 days (1), 42 days (1), 49 days (2) and 56 days (2) of age.

Sites examined

Routine aerobic and microaerophilic cultures were carried out on appropriate tissues of animals which died unexpectedly or where there was evidence of abnormality grossly suggesting an infectious cause. All other examinations were specifically attempts to recover mucosalis, and for the majority of animals samples were taken from the three mucosal sites (M.S.I., T.S.I. and L.I.) and in certain instances from the mouths. Control piglets, sacrificed to demonstrate freedom from infection with mucosalis before the remainder were exposed, were examined in a similar fashion. Unexplained deaths were also sampled for mucosalis, where the preservation of the tissues and other factors suggested that the information which this might yield could be of value. Appropriate adjacent mucosal samples for electron microscopy, immunofluorescence and light microscopy were taken from piglets at necropsy.

Bacteriological examination

Mucosal samples were examined quantitatively in the normal manner using 1/20 serial dilutions plated out in 0.1 ml amounts on C.B.A. or N.B.G. Chyme, where examined, was plated out conventionally on inhibitory and non-inhibitory media.

Calculation of CUSUM values

The figures for body weight were treated as described in Chapter III, Part C.

$$\text{CUSUM} = \text{gain in body weight} - 2.0$$

No figures for weight gains in piglets of this age were available so the constant 2.0 was chosen arbitrarily.

RESULTS

In an attempt to assist the reader to follow the experiments the results of the clinical, pathological, bacteriological and immunofluorescent examinations of each of these three litters are brought together.

Litter ex Sow F

Clinical Findings/Daily Observations

At twelve hours post-dosing two piglets were found dead, neither having shown previous signs of illness. A further piglet was found dead and another dying at twenty four hours post-dosing. By one week a number of the piglets were noticeably smaller, and this was true of one in particular (pig 108/76), which although weak was still suckling, and was not diarrhoeic. This piglet became progressively weaker and was killed on humane grounds at twelve days post-dosing, when it weighed only 2.75 lbs in weight. From thirteen days of age the piglets were weighed approximately weekly and the CUSUM values calculated (see Table 7b). All the remaining piglets were alert, bright and ate well, and at thirty five days post exposure two piglets were selected for euthanasia (133/76 and 134/76), since they had the lowest CUSUM values.

Although the two pigs killed at thirty five days post-dosing appeared to be eating and showed no other signs of disease, one had lost 0.5 lb and the other 1 lb in body weight over the previous seven days, representing a fall of 10% and 14% respectively, in the body weights of these two animals. From about that time pig 141/76 became anorexic and showed little or no interest in food.

Table 7b Body Weights and CUSUM Values for the Litter ex Sow F.

Pig Reference	13		27		34		41		47		61		69		76	
	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C
108/76	2.75															
133/76	5.00		5.50	-3.50	5.00	-6.00										
134/76	6.00		8.00	-2.00	7.00	-5.00										
141/76	6.00		10.00	0	8.50	-3.50	7.50	-6.50								
190/76	8.50		13.00	0.50	11.00	-3.50	11.00	-5.50	14.00	-4.50	18.00	-4.50				
209/76	4.50		8.50	0	10.00	-0.50	11.00	-1.50	12.50	-2.00	17.00	-1.50	21.00	0.50		
216/76	7.50		13.50	2.00	14.00	0.50	16.00	0.50	16.50	-1.00	20.00	-1.50	24.00	0.50	23.00	-2.50
217/76	7.00		11.00	0	11.00	-2.00	12.00	-3.00	14.00	-3.00	19.00	-2.00	23.00	0	27.00	2.00
219/76	5.50		10.25	0.75	11.00	-0.50	12.50	-1.00	14.50	-1.00	22.00	2.50	25.00	3.50	30.00	6.50

W = Body weight in lbs

C = CUSUM value

At 27 days and 61 days post-dosing there had been an interval of approximately two weeks since the previous weighing and CUSUM = gain in body weight - 4.0.

It was thin, but alert and was killed at forty two days post-dosing. In the seven days preceding euthanasia, this piglet had lost 1 lb in body weight, i.e., approximately 13% of its body weight. The remainder of the litter showed no signs of disease throughout the experiment, and appeared to eat the creep feed offered. They were selected for euthanasia on their CUSUM values, the pigs with the lowest values being selected. From Table 7b it can be seen that despite the fact that no signs of disease were noted in these piglets a number either lost weight or gained no weight between two successive weighings on a number of occasions. It is stressed that these piglets were offered meal ad-lib.

Gross Pathology/Histopathology

The findings at necropsy, and the histopathological observations on the piglets of the litter of sow F, are listed in Table 7c. Pertinent features will now be considered in more detail.

None of these piglets showed gross changes of PIA. In only one piglet (190/76) was adenomatous change seen histologically. In the L.I. of a number of these pigs there were worm larvae in the mucosa, with an associated lymphocytic infiltration. In the pigs killed at the end of the experiment *Trichuris* worms were present on the surface of the large intestinal mucosa. Most of the piglets killed after twelve days of age showed changes in the L.I. manifest as a colitis of varying severity or damage to the epithelium, which was less well stained and preserved. Associated with these changes the large intestinal crypts were seen to contain a large bacterial population. This was seen in H. and E. and silver-stained sections,

Table 7c Gross-Pathology/Histopathology - Litter ex Sow F.

Pig Reference	Gross Pathology	Histopathology
74/76	Cyanosis skin and ears Intestines flaccid.	Congestion liver, kidneys and lungs. Alimentary tract N.A.D.
75/76	No gross lesions	Congestion liver, lungs, spleen. Alimentary tract N.A.D.
80/76 and 81/76	Stomach empty, Intestines empty and congested. meconium voided.	Alimentary tract N.A.D.
108/76	Right apical, left and right diaphragmatic lobes of lung - a number of pneumonic foci. Stomach empty, Intestines - a number of gas-filled loops.	Lungs - bronchopneumonia Alimentary tract - M.S.I. - total villous atrophy, and crypt hyperplasia. Neutrophils in the lamina propria.
133/76 and 134/76	No gross lesions	M.S.I. T.S.I. - villous atrophy. L.I. - colitis.
141/76	N.A.D.	M.S.I., T.S.I. - villous atrophy. L.I. - colitis, and occasional helminth larvae in the mucosa.
190/76	N.A.D.	M.S.I. - villous atrophy, large numbers of eosinophils in the lamina propria. T.S.I. - occasional adenomatous glands.
209/76 and 216/76	N.A.D.	M.S.I., T.S.I., - villous atrophy. L.I. - some epithelial damage, + eosinophils.
217/76 and 219/76	N.A.D. Occasional Trichuris worms in the large intestine	As 209/76

N.A.D. = No abnormality detected.

and the bacteria were of mixed morphology, some vibrios were recognised, but no spirochaetes were noted.

Pig 108/76

In the M.S.I. of this piglet there was total villous atrophy, much of the surface epithelium being cuboidal to flattened. Neutrophils were present under the surface epithelium, and throughout the lamina propria. They were also present within the epithelium and gland lumina in some glands. There were few goblet cells present, and no frankly adenomatous glands were seen. The changes in the T.S.I. were similar but less severe. In silver-stained sections no intracellular vibrios were observed.

In the L.I. in many of the gland lumina there were accumulations of mucus and degenerating neutrophils, and sometimes associated with this the glandular epithelium was considerably flattened. Around these glands the lamina propria contained neutrophils, which were also seen intra-epithelially. Some of the crypts had a crowded appearance, but more superficially in the mucosa there were abundant goblet cells. Intracellular bacteria were not visualised in silver-stained sections.

The changes in this piglet to some extent resemble those described in the "three week scours" animals in Chapter III, Part D, and also in pig 123/76 from the litter of sow G, this chapter.

Pig 190/76

In the M.S.I. there was villous atrophy and crypt hyperplasia, and an increase in the cellularity of the lamina propria,

due especially to eosinophils, which were present in very large numbers. In the T.S.I. the histological appearance was similar, but in this site occasional adenomatous glands were seen. These glands appeared to be "growing out" of the mucosa. They were situated more superficially in the mucosa and the underlying glands were of normal morphology. Surrounding these adenomatous glands there was an increased number of cells in the lamina propria, due to an increase in both lymphocytes and neutrophils. Neutrophils were also present within the epithelium and there was cell debris and neutrophils present within the lumina. These changes are consistent with the recovery phase of PIA (Chapter IX). In these adenomatous gland cells bacteria of vibrio morphology were demonstrated in silver stained sections. The L.I. was unremarkable.

Bacteriological Results

The recovery of mucosalis from the piglets in the litter of sow F is presented in Table 7d.

Mucosalis was recovered from the M.S.I. mucosa of piglet 219/76, killed at seventy six days of age. This piglet had been dosed at birth and twenty hours post-partum, and it had also been mixed and housed with a group of piglets (litter of sow G), a number of which had PIA, from forty seven days of age.

The isolations of mucosalis from the three pigs in this litter were made from the mucosal samples listed below on the media indicated:

108/76	T.S.I.	$(\frac{1}{20})^2$	dilution on N.B.G.
134/76	L.I.	$(\frac{1}{20})^2$	dilution on N.B.G.
219/76	M.S.I.	$(\frac{1}{20})^2$	dilution on N.B.G. and B.A.

Table 7d Results of Bacteriological and Immunofluorescence Examination of the Piglets in the Litter of Sow F.

Pig Reference	Age	Bacteriology				Immunofluorescence		
		Mouth	M.S.I.	T.S.I.	L.I.	M.S.I.	T.S.I.	L.I.
74/76	12 hrs	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
75/76	12 hrs	N.D.	-	-	-	N.D.	N.D.	N.D.
80/76	24 hrs	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
81/76	24 hrs	N.D.	-	-	N.D.	N.D.	N.D.	N.D.
108/76	12 dys	-	-	+ ¹	-	-	-	-
133/76	35 dys	-	-	-	-	-	-	++/-- lumen
134/76	35 dys	-	-	-	+ ²	-	-	++/-- lumen
141/76	42 dys	-	-	-	-	++/-- lumen	-	++/-- lumen
190/76	64 dys	-	-	-	-	++/-- lumen	-	-
209/76	70 dys	-	-	-	-	-	-	-
216/76	70 dys	-	-	-	-	-	-	++/--
217/76	76 dys	-	-	-	-	-	-	-
219/76	76 dys	-	+ ³	-	-	-	-	++/-- lumen

++/-- Bright, particulate fluorescence but not in large quantities
N.D. = Not done

¹ $\geq 8 \times 10^4$ viable mucosalis per gram of mucosa

² $\geq 4 \times 10^3$ viable mucosalis per gram of mucosa

³ 8×10^4 viable mucosalis per gram of mucosa

Bordetella bronchiseptica was isolated from the lungs of pig 108/76.

Immunofluorescence Results

The results of the immunofluorescence examination carried out on the piglets of the litter of sow F are listed in Table 7d.

Electron Microscopic Results

Material from a number of piglets in the litter of sow F was examined, particular attention being directed towards those pigs from which mucosalis had been isolated, and the sites from which recovery of mucosalis had been achieved. No intracellular vibrios or other bacteria were seen. In the lumina of some glands in the large intestine bacteria were present, this population contained a number of morphological types of organisms, some of which morphologically resembled mucosalis. It is not possible to differentiate mucosalis from ultrastructurally similar organisms in this site by electron microscopy.

Litter ex Sow G

Clinical Findings /Daily Observations

The piglets initially appeared unaffected by exposure to infection, and at nine days were evenly matched in weight (Table 7e). From the end of the second week it became noticeable that piglet 123/76 was smaller in comparison to its litter mates, and it appeared to show less interest in food. In the third week after dosing size differences between the other piglets were evident. At twenty three days post-dosing pig 123/76 was killed, having lost

Table 7e Body Weights and CUSUM Values for the Litter ex Sow G.

Pig Reference	8		22		29		36		42		56		64	
	Days Post-dosing													
	W	C	W	C	W	C	W	C	W	C	W	C	W	C
123/76	5.25		4.00	-5.25										
140/76	6.00		10.25	0.25	8.50	-3.50	6.00	-8.00						
156/76	7.00		12.50	1.50	12.50	-0.50	16.00	1.00	15.00	-2.00				
157/76	7.50		12.25	0.75	10.50	-3.00	10.50	-5.00	9.00	-8.50				
183/76	4.75		9.00	0.25	7.50	-3.25	7.50	-5.25	6.50	-8.25	8.50	-10.25		
189/76	6.00		9.00	-1.00	8.50	-3.50	8.50	-5.50	9.50	-6.50	12.00	-8.00		
206/76	5.50		8.75	-0.75	7.50	-4.00	8.00	-5.50	7.50	-8.00	13.00	-6.50	14.00	-7.50
207/76	7.50		12.00	0.50	9.50	-4.00	9.50	-6.00	10.50	-7.00	15.00	-6.50	17.00	-6.50
208/76	6.00		9.50	-0.50	8.50	-3.50	10.50	-3.50	11.50	-4.50	16.00	-4.00	19.00	-3.00

W = Body weight in lbs

C = CUSUM value

At 22 days and 56 days post-dosing there had been an interval of approximately two weeks since the previous weighing and CUSUM = gain in body weight - 4.0.

1.25 lbs over the previous fourteen days, this representing a loss of approximately 24% in body weight. At euthanasia pig 123/76 was weak, but not diarrhoeic. During the fourth and fifth weeks post-dosing the piglets had variable appetites, were less active and alert than previous litters kept under similar conditions. A number of the piglets in the litter, although well grown, were thin with prominent vertebral processes. At the end of the fifth week, thirty seven days post-dosing pig 140/76 was killed. It was selected because it had the lowest CUSUM value at that time, and it had lost 4.25 lbs in weight, from weighing 10.25 lbs at twenty two days post-dosing. This represents a loss of approximately 41% in body weight over the two weeks. Up to forty three days of age, when they were mixed with the remaining piglets from the litter of sow F, the rest of the litter were less active and alert, there was considerable size variation, and they showed variable appetites. The piglets would rush to the trough immediately that the meal was changed and stand with their heads down, but if observed closely not all of them would actually be eating.

After mixing with the remaining piglets of sow F it was noticed that piglet 156/76 was definitely anorexic. It stood apart from the others, while they rushed to the trough. It would occasionally stand at the trough with the others but did not eat. At fifty days post-dosing pig 156/76 was weaker and showed obvious pain if handled. On humane grounds euthanasia was carried out at that time. At fifty two days post infection pig 157/76 was killed, selection being based on the fact that it had the lowest CUSUM value at the most recent weighing.

During the eighth week post-dosing the remaining pigs appeared to have improved appetites, food consumption increased and they were generally brighter and more alert. Selection of pigs was based on CUSUM values, those with the lowest values being chosen on each occasion.

Gross Pathology/Histopathology

The gross and histopathological findings of the piglets in the litter of sow G are listed in Table 7f. Any changes of PIA seen in the piglets of the litters of sows F and H were minimal. In contrast almost all the piglets in the litter of sow G showed evidence of PIA. The changes seen in these piglets will now be considered more fully.

Figs 85/76 and 86/76: Both these piglets were killed before dosing of their litter mates, and no changes were detected in the alimentary tracts.

Fig 123/76 (23 days post-dosing): This piglet was in very poor bodily condition, but no gross lesions were noted in the alimentary tract or elsewhere in the carcass. There was no histological evidence of adenomatous change, but in the M.S.I. and T.S.I. there was severe villous atrophy resulting in flattening of the mucosal surface, the crypts although not greatly lengthened had an increased cellularity (Figure 59). In the L.I. there was some surface damage associated with balantidia, and in some gland lumina there was cell debris. Much of the epithelium was cuboidal with few goblet cells present, but the glands were not adenomatous although some had a crowded appearance (Figure 60). Intracellular vibrios were not seen in silver stained sections.

Table 7f Gross Pathology/Histopathology - Litter ex Sow G.

Pig Reference	Gross Pathology	Histopathology
85/76 and 86/76	N.A.D.	N.A.D.
123/76	M.S.I., T.S.I. - Little content, some loops distended with gas. L.I. - tenacious, grey contents with mucus consistency.	M.S.I., T.S.I. - Severe villous atrophy. L.I. - glands lined by cuboidal epithelium. Cell debris in lumina.
140/76	L.I. - enlarged, with a reticulated appearance to the serosal surface. Exaggerated folding of the mucosa with necrosis of the tips.	M.S.I., T.S.I. - villous atrophy. L.I. - underlying the necrosis there were adenomatous glands.
156/76	M.S.I., T.S.I. - NEL L.I. - PIA	M.S.I., T.S.I. - confirms that NE. L.I. - confirms that PIA.
157/76	N.A.D., except for slight thickening of L.I.	M.S.I., T.S.I. - villous atrophy, and occasional isolated adenomatous glands. L.I. - hyperplastic, not adenomatous.
183/76 and 189/76	T.S.I. and L.I. - PIA	M.S.I. - isolated adenomatous glands. T.S.I. - PIA L.I. - isolated adenomatous glands.
206/76, 207/76 and 208/76	N.A.D. except for the L.I. of pig 207/76 which was finely, but visibly, thrown into folds.	M.S.I., T.S.I. - villous atrophy, with isolated adenomatous glands. L.I. - occasional adenomatous glands.

N.A.D. = No abnormality detected.

Pig 140/76 (37 days post-dosing): At necropsy there were no gross changes in the small intestine, and histologically the only changes were of villous atrophy and crypt hyperplasia, similar to those seen in pig 123/76. The large intestine was enlarged and had a reticulated appearance from the serosal surface, the mucosa was thickened and thrown into exaggerated folds (Figure 61). The tips of the folds of mucosa were necrotic (Figure 61). Histologically in the L.I. some areas of the mucosa had elongated crypts and a substantial goblet cell population. In the damaged necrotic areas there were large numbers of balantidia, and the surviving glandular elements had an adenomatous appearance (Figures 62 and 63). Few balantidia were seen in those areas with large numbers of mucus-secreting cells. On occasion there was sufficient damage to the epithelium to result in ulceration, with granulation tissue proliferation in the lamina propria and sub-mucosa.

Intracellular vibrios were not seen in silver stained sections, which, as mentioned previously, were found difficult to interpret if the bacteria were only present in low numbers or if the tissue was damaged.

Pig 156/76 (50 days post-dosing): The terminal one metre of the small intestine of this piglet was thickened and showed changes typical of NE (Figure 64). In the large intestine, the mucosa of the caecum and proximal twenty centimetres of the colon showed thickening typical of PIA (Figure 64). Histologically the changes in the small intestine were confirmed as those of NE, with coagulative necrosis of an already adenomatous mucosa (Figures 65 and 66).

Intracellular vibrios were demonstrated in the apical cytoplasm of the epithelial cells of the surviving glandular elements. The changes in the L.I. were confirmed as typical of PIA, and the characteristic intracellular bacteria were demonstrated in silver-stained sections. In some areas of the L.I. the mucosa was frankly adenomatous, while in others there were adenomatous glands and dilated glands, with a flattened cuboidal epithelium interspersed (Figure 67). In these latter areas the shape of glands was very varied.

Pigs 183/76 and 189/76 (58 days post-dosing): These two pigs are considered together as the changes present were essentially similar. The terminal metre of the small intestine had a reticulated appearance from the serosal surface, and the mucosa was thickened. There was an exaggerated folding of the mucosa over Peyer's patches, and also involving the non-lymphoid areas (Figure 68). The large intestinal mucosa was also thickened and thrown into folds, but these were less prominent than in the small intestine (Figure 69). The caecum and approximately the proximal one metre of the colon were involved.

Histologically in the M.S.I. there were isolated adenomatous glands (Figure 70). These were larger than the surrounding normal glands, and lined by large, immature epithelial cells, although some of the glands contained a number of goblet cells. In the T.S.I. the mucosa had a typical adenomatous appearance, with loss of villous architecture and in some of the deeper glands occasional goblet cells were present. In the L.I. adenomatous glands were present in

the mucosa, but many of these glands contained a variable number of goblet cells (Figure 71). Only isolated adenomatous glands were present in some areas, the surrounding mucosa having a substantial goblet cell population (Figure 72). In the adenomatous glands in the mucosa of all three sites intracellular vibrios were demonstrated in silver-stained sections.

Figs 206/76, 207/76 and 208/76 (64 days, 64 days and 65 days post-dosing): Except for the large intestine of pig 207/76, there were no gross changes seen in these piglets. The large intestinal mucosa of pig 207/76 was minutely, but visibly thrown into exaggerated folds.

Histologically in these cases there was villous atrophy and crypt hyperplasia in the mucosa of the M.S.I. and T.S.I. (Figure 73). There were also occasional isolated adenomatous glands present (Figure 73). These glands were situated superficially in the mucosa, and appeared to be growing out of the mucosa, the underlying glands were normal in appearance (Figure 74). The large intestinal mucosa of these pigs showed crypt hyperplasia and disordered gland morphology but only a few glands were definitely adenomatous (Figure 75). There was a fairly substantial goblet cell population within the large intestinal epithelium, and the lamina propria had increased numbers of cells, lymphocytes, macrophages and large numbers of plasma cells.

Bacteriological Results

The recovery of mucosalis from the litter of sow G is presented in Table 7g. Mucosalis was recovered from piglets in

Table 7g Results of Bacteriological and Immunofluorescence
Examination of the Piglets in the Litter of Sow G.

Pig Reference	Age	Bacteriology				Immunofluorescence		
		Mouth	M.S.I.	T.S.I.	L.I.	M.S.I.	T.S.I.	L.I.
85/76	*	N.D.	N.D.	-	-	N.D.	-	-
86/76	*	N.D.	N.D.	-	-	N.D.	-	-
123/76	23 dys	-	-	+ ¹	+ ²	+	+	+
140/76	37 dys	-	+ ³	+ ⁴	+ ⁵	+	+	+
156/76	50 dys	N.D.	+ ⁶	+ ⁷	+ ⁸	+	+	+
157/76	52 dys	-	-	-	-	+	+	-
183/76	58 dys	-	-	-	-	+	+	+
189/76	58 dys	-	-	+ ⁹	+ ¹⁰	+	+	+
206/76	64 dys	-	-	-	-	+	+	+
207/76	64 dys	-	-	-	-	+	+	+
208/76	64 dys	-	-	-	-	+	+	+

* Killed before dosing

N.D. = Not done.

1	$\geq 1.34 \times 10^4$	viable <u>mucosalis</u> per gram of mucosa						
2	8.0×10^4	"	"	"	"	"	"	"
3	4.0×10^3	"	"	"	"	"	"	"
4	$\geq 1.6 \times 10^3$	"	"	"	"	"	"	"
5	2.08×10^8	"	"	"	"	"	"	"
6	3.2×10^8	"	"	"	"	"	"	"
7	$\geq 3.84 \times 10^7$	"	"	"	"	"	"	"
8	1.3×10^8	"	"	"	"	"	"	"
9	1.12×10^5	"	"	"	"	"	"	"
10	$\geq 6.8 \times 10^4$	"	"	"	"	"	"	"

this litter up to and including the fifty eighth day after dosing. Mucosalis was not recovered from the oral cavities of any of these piglets. The numbers of mucosalis recovered from these pigs is also recorded in Table 7g. The mucosal samples from which mucosalis was recovered, and the media on which recovery was made, are listed below.

- Pig 123/76 T.S.I. on N.B.G. at the $\frac{1}{20}$ dilution.
 L.I. on N.B.G. at the $(\frac{1}{20})^2$ dilution and on B.A. at $(\frac{1}{20})^3$ dilution.
- Pig 140/76 M.S.I. on N.B.G. at $\frac{1}{20}$ dilution and on B.A. at $(\frac{1}{20})^2$ dilution.
 T.S.I. on N.B.G. at $\frac{1}{20}$ dilution.
 L.I. on N.B.G. and B.A. at $(\frac{1}{20})^2$, $(\frac{1}{20})^3$, and $(\frac{1}{20})^4$ dilutions.
- Pig 156/76 M.S.I. on N.B.G. at $(\frac{1}{20})^3$ dilution and on B.A. at $(\frac{1}{20})^4$ dilution.
 T.S.I. on N.B.G. at $(\frac{1}{20})^3$ dilution.
 L.I. on N.B.G. at $(\frac{1}{20})^3$ dilution and on B.A. at $(\frac{1}{20})^4$ dilution.
- Pig 189/76 T.S.I. on N.B.G. and C.B.A. at $(\frac{1}{20})^2$ dilution.
 L.I. on N.B.G. at $\frac{1}{20}$ and $(\frac{1}{20})^2$ dilutions.

Immunofluorescence Results

The results of the immunofluorescence examination of tissues from the piglets in the litter of sow G are summarised in Table 7g. The results in individual piglets in this litter will now be considered in greater detail.

Pig 123/76 (23 days post-dosing): In all three sites, M.S.I., T.S.I. and L.I., there was positive particulate fluorescence present within the apical cytoplasm of some cells in occasional glands. There was only a little fluorescence per cell, much less than in the positive control sections stained in parallel.

Pig 140/76 (37 days post-dosing): The staining in the M.S.I., and T.S.I. of this piglet was similar to that in pig 123/76, some cells in occasional glands containing a little intracellular fluorescence, located in the apical cytoplasm. In the L.I. there was bright positive fluorescence in the glands underlying the areas of necrosis. These glands stained almost as brightly as the positive controls, and the fluorescence was again limited to the apical cytoplasm. In H. and E. stained sections these glands had an adenomatous appearance. The surrounding normal glands, with a substantial goblet cell population did not show fluorescence.

Pig 156/76 (50 days post-dosing): In the M.S.I. and T.S.I. the underlying intact glands showed bright positive fluorescence, located in the apical cytoplasm of the cells. This was as bright, and as abundant as the fluorescence in the control cases stained in parallel. In the overlying necrotic tissue positive particulate fluorescence was also seen within the outlines of necrotic cells. The adenomatous epithelial cells in the glands of the L.I. were also positive for mucosalis antigen.

Pig 157/76 (52 days post-dosing): In the M.S.I. and T.S.I. in occasional glands there was bright particulate fluorescence, these glands corresponded to the adenomatous glands seen in H. and E.

stained sections. The surrounding normal glands did not fluoresce. The L.I. did not show fluorescence, and parallel sections stained with H. & E. did not show evidence of PIA.

Figs 183/76 and 189/76 (58 days post-dosing): In all three sites, M.S.I., T.S.I. and L.I., there was some positive particulate fluorescence in the apical cytoplasm of the epithelial cells. This was however less than expected from the number of adenomatous glands seen in parallel sections stained with H. and E., and from the fluorescence seen in positive controls stained at the same time. Some adenomatous glands did not stain at all and in others the fluorescence was more diffuse in character than usual. Occasional particles of fluorescence were seen to be more basal in position, rather than the apical cytoplasmic distribution usually seen. There were cells present in the gland lumina which contained positive fluorescence and also free fluorescing organisms. In the lamina propria there were cells containing mucosalis antigen which fluoresced. In these cells, which light microscopy indicated to be macrophages, the fluorescence was arranged mostly around the periphery of the cell.

Figs 206/76, 207/76 and 208/76 (64 days, 64 days and 65 days post-dosing): In all three sites, M.S.I., T.S.I. and L.I., there were some glands which contained positive fluorescence, although this was often only present in a proportion of the cells in the gland, and was usually only a small quantity per cell. Not all the glands which were adenomatous, in parallel sections stained by H. and E., showed positive fluorescence. There were cells present within the gland lumina which contained positive fluorescence and free fluorescing vibrios were also seen. In the lamina propria there were large numbers

of fluorescing cells, this mucosalis antigen was arranged mostly around the periphery of the cells, which were probably macrophages.

Electron Microscopy

Electron microscopic examination was undertaken on tissues from all of the pigs in the litter of sow G and the results are presented below.

Pigs 123/76 and 140/76 (23 days and 37 days post-dosing):

Intracellular bacteria were not seen in tissue from any of the sites examined from these pigs.

Pig 156/76 (50 days post-dosing):

Large numbers of curved bacterial bodies were present in the apical cytoplasm of the epithelial cells in the mucosa of the M.S.I. T.S.I. and L.I. These bacteria resembled mucosalis and were free within the cytoplasm and not surrounded by host cell membranes. This applied to the intact mucosa underlying the more superficial necrotic mucosa in the M.S.I. and T.S.I. In the areas where the mucosa had undergone coagulative necrosis the invading bacteria were markedly pleomorphic and where these had invaded degenerating epithelial cells the bacterial population present in the cell was very mixed.

Pig 157/76 (52 days post-dosing): In the isolated adenomatous glands present within the mucosa of the M.S.I. and T.S.I. of this pig, bacteria, typical of mucosalis were seen free within the apical cytoplasm of the epithelial cells. In the surrounding normal glands no bacteria were seen within the epithelium.

Pigs 183/76 and 189/76 (58 days post-dosing): The tissue examined ultrastructurally from these two pigs, from the affected

areas of both the small intestine and large intestine conformed to the descriptions already given for PIA (Chapter III). Bacteria, resembling mucosalis, were seen free within the apical cytoplasm of the immature epithelial cells. In addition, however, there were a number of features indicative that these animals were entering the recovery phase of PIA (Chapter IX). As well as free within the cytoplasm bacteria were also seen within membrane-bound vesicles in the intestinal epithelial cells. Bacteria, indistinguishable from mucosalis, were also present within macrophages and polymorphonuclear leucocytes. Some of the bacteria in the intestinal epithelial cells were more osmiophilic, had a shrunken appearance and the outer wavy membranes were less distinct.

Figs 206/76, 207/76 and 208/76 (64 days, 64 days and 65 days post-dosing): In those areas in which adenomatous glands were present the ultrastructural changes resembled those described for pigs 183/76 and 189/76. In some of the adenomatous glands there were occasional epithelial cells which were more mature and had better developed brush borders. These latter cells had only a few or no bacteria present. Goblet cells were also seen in some of the glands.

Litter ex Sow H

Clinical Findings /Daily Observations

As this sow showed post-parturient agalactia the piglets were offered milk substitute in addition to creep for the first ten days after birth. At the end of the second week the piglets could be seen to vary in size and a number of them were noticeably thinner. The piglets were weaned at twenty two days of age (twenty one days

post-dosing) and weighed weekly from thirteen days of age (see Table 7h). Throughout the duration of the experiment the piglets always showed an interest in food, but as in the litter of sow G, little food appeared to be eaten.

From Table 7h it can be seen that the pig killed at thirty three days post-dosing (pig 241/76) had failed to gain any weight between thirteen days and twenty eight days post-dosing. Similarly, for pig 251/76, killed at forty one days post-dosing, the weight at thirteen days was identical to that at thirty nine days post exposure. The pigs killed at forty eight days post-dosing were selected on their CUSUM values. At this time pig 263/76 had gained only 3.5 lbs weight over the previous thirty three days, and pig 264/76 had gained 7 lbs over this same period. The pigs killed at fifty five days post-dosing had gained 10 lbs and 11 lbs over the previous forty days.

Gross Pathology/Histopathology

The findings in the litter of sow H are presented in Table 7j. In none of the piglets in the litter of this sow was there definite gross evidence of PIA. Histopathological changes of PIA were only seen in one case, in pig 251/76, in the T.S.I. In a number of the piglets there was evidence of damage to the mucosa of the large intestine. There were no helminth larvae or adults seen in any of the animals. Balantidia were present in the large intestines of a few of the piglets. The damage to the large intestine presented either as a colitis or detachment of the surface epithelium.

Table 7h Body Weights and CUSUM Values for the Litter ex Sow H.

Pig Reference	13		21		28		39		46		53	
	W	C	W	C	W	C	W	C	W	C	W	C
241/76	6.00		6.00	-2.00	6.00	-4.00						
251/76	7.00		7.00	-2.00	7.00	-4.00	7.00	-7.00				
263/76	8.00		9.00	-1.00	9.00	-3.00	11.00	-4.00	11.50	-5.50		
264/76	9.00		12.00	1.00	12.00	-1.00	15.00	-1.00	16.00	-2.00		
275/76	10.00		12.00	0	14.00	0	16.00	-1.00	18.00	-1.00	20.00	-1.00
276/76	9.00		12.00	1.00	12.00	-1.00	15.00	-1.00	17.00	-1.00	20.00	0

W = Body weight in lbs.

C = CUSUM value.

At 39 days post-dosing there had been an interval of 11 days since the previous weighing and CUSUM = gain in body weight - 3.0.

Table 7j Gross Pathology/Histopathology - Litter ex Sow H.

Pig Reference	Gross Pathology	Histopathology
174/76 and 175/76	Stomach and small intestines empty. Meconium present in L.I.	N.A.D.
173/76a, and 173/76b	Dehydrated. Alimentary tract empty.	N.A.D.
241/76	M.S.I., T.S.I. Peyer's patches very prominent. L.I. - serosal surface - reticulated appearance, but mucosa not thickened.	M.S.I., T.S.I. - villous atrophy. L.I. - colitis.
251/76	N.A.D.	M.S.I., T.S.I. - villous atrophy, and in the T.S.I. also occasional isolated adenomatous glands.
263/76	N.A.D.	M.S.I., T.S.I. - villous atrophy.
264/76	L.I. - possible thickening of the mucosa.	L.I. - colitis, surface epithelial damage, and + balantidia.
275/76	N.A.D.	M.S.I., T.S.I. - villous atrophy. Large numbers of eosinophils in the lamina propria.
276/76	N.A.D.	M.S.I., T.S.I. - villous atrophy. L.I. - surface epithelium detached, balantidia present.

N.A.D. = No abnormality detected.

Bacteriological Results

The bacteriological results for the piglets in the litter of sow H are presented in Table 7k. Mucosalis was recovered from only one piglet (251/76) in this litter, at forty three days post-dosing. The single isolation of mucosalis which was made from this pig was from the 1/20 dilution T.S.I. mucosal sample on N.B.G. Mucosalis was not recovered from the oral cavities of any of the piglets in this litter.

Immunofluorescence Results

The results of immunofluorescence examination of tissues from the piglets of the litter of sow H are summarised in Table 7k.

Electron Microscopy

Only a small number of tissues from pigs in the litter of sow H were examined. No intracellular bacteria were seen in the intestinal epithelium. Occasionally a crypt population of bacteria were seen in the large intestine, this was made up of a number of morphological types of organisms and mucosalis could not be identified.

Summary

The results of these various investigations on the piglets in the litters of sows F, G and H are summarised in Tables 7m, 7n and 7p respectively.

Table 7k Results of Bacteriological and Immunofluorescence Examination of the Piglets in the Litter of Sow H.

Pig Reference	Time Post-dosing	Bacteriology				Immunofluorescence		
		Mouth	M.S.I.	T.S.I.	L.I.	M.S.I.	T.S.I.	L.I.
174/76	18 hrs	N.D.	-	N.D.	N.D.	N.D.	N.D.	N.D.
175/76	18 hrs	N.D.	-	N.D.	N.D.	N.D.	N.D.	N.D.
173/76b	36 hrs	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
173/76a	60 hrs	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
241/76	35 dys	-	-	-	-	-	-	++/-- lumen
251/76	43 dys	-	-	+ ¹	-	-	-	++/-- lumen
263/76	50 dys	-	-	-	-	-	-	-
264/76	50 dys	-	-	-	-	-	-	-
275/76	57 dys	- ²	-	-	-	-	-	-
276/76	57 dys	- ²	-	-	-	-	-	++/-- lumen

++/-- Bright, particulate fluorescence but not in large quantities.

N.D. = Not done.

¹ $\gg 2.4 \times 10^3$ viable mucosalis per gram of mucosa.

² Other catalase negative, slide agglutination negative campylobacters present in the mouth.

Table 7m Summary of Results - Litter ex Sow F.

Pig Reference	Evidence of PIA/NE		Mucosalis Demonstrated			
	Gross	Histology	Silver-staining	Electron-microscopy	Bacteriology	Immunofluorescence
108/76	-	-	-	-	+T.S.I.	-
133/76	-	-	-	-	-	+L.I.
134/76	-	-	-	-	+L.I.	+L.I.
141/76	-	-	-	-	-	+M.S.I., L.I.
190/76	-	+T.S.I.	+T.S.I.	N.D.	-	+/-M.S.I.
209/76	-	-	-	N.D.	-	-
216/76	-	-	-	N.D.	-	+/- L.I.
217/76	-	-	-	N.D.	-	-
219/76	-	-	-	N.D.	+M.S.I.	+L.I.

N.D. = Not done.

Table 7n Summary of Results - Litter ex Sow G.

Pig Reference	Evidence of PIA/NE		Mucosalis Demonstrated			
	Gross	Histology	Silver-staining	Electron-microscopy	Bacteriology	Immunofluorescence
123/76	-	-	-	-	+T.S.I., L.I.	+M.S.I., T.S.I., L.I.
140/76	+L.I.	+L.I.	-	-	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.
156/76	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+T.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.
157/76	-	+M.S.I., T.S.I.	+M.S.I., T.S.I.	+M.S.I., T.S.I.	-	+M.S.I., T.S.I.
183/76	+T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	-	+M.S.I., T.S.I., L.I.
189/76	+T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+T.S.I., L.I.	+M.S.I., T.S.I., L.I.
206/76	-	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	N.D.	-	+M.S.I., T.S.I., L.I.
207/76	(+L.I.)	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	-	+M.S.I., T.S.I., L.I.
208/76	-	+M.S.I., T.S.I., L.I.	+M.S.I., T.S.I., L.I.	N.D.	-	+M.S.I., T.S.I., L.I.

N.D. = Not done.

Table 7p Summary of Results - Litter ex Sow H.

Pig Reference	Evidence of PIA/NE		Mucosalis Demonstrated			
	Gross	Histology	Silver-staining	Electron-microscopy	Bacteriology	Immunofluorescence
241/76	-	-	-	-	-	+/- L.I.
251/76	-	+T.S.I.	-	-	+T.S.I.	+/- L.I.
263/76	-	-	-	N.D.	-	-
264/76	-	-	-	N.D.	-	-
275/76	-	-	-	N.D.	-	-
276/76	-	-	-	N.D.	-	+ L.I.

N.D. = Not done.

Recovery of *mucosalis* on inhibitory and non-inhibitory media

If one considers the recovery of *mucosalis* from the piglets in all three litters, with respect to the media on which isolation was made the following are the results.

Isolation on N.B.G. and B.A., at the same mucosal dilution	3
Isolation on N.B.G. and B.A., at different mucosal dilution	4
Isolation on N.B.G. only	7

In half of the sites from which *mucosalis* was isolated, recovery was made from the N.B.G. plates only and not from B.A. plates inoculated in parallel. This was especially true of those sites from which *mucosalis* was only recovered in small numbers. Also in one site from pig 156/76, which had N.E., overgrowth by other bacteria on the B.A. plates precluded recovery of *mucosalis*, and isolation was made on the N.B.G. plates. Where *mucosalis* was recovered in large numbers, recovery was usually made on both B.A. and N.B.G. plates. The overgrowth by other bacteria on the non-inhibitory media usually meant that recovery on B.A. could only be made from higher dilutions of the mucosa, whilst N.B.G. permitted recovery from lower dilutions.

DISCUSSION

The transmission experiments conducted were successful and there was evidence of PIA in some piglets from all three litters. In the litter of sow F there was histological evidence of PIA in one pig, and *mucosalis* was recovered from three others, and in the litter of sow H there was histological evidence of PIA in only one

pig and mucosalis was isolated from this same animal. In the litter of sow G, a greater degree of success was achieved and eight of the nine piglets had evidence of PIA, and mucosalis was recovered from the ninth piglet. Although PIA was not seen in this last piglet the lesions were possibly at the pre-adenomatous stage.

Fortuitously the piglets in the litter of sow G were killed over a wide range encompassing the period before development of adenomatous lesions, the stage at which gross and histological evidence of PIA was present and mucosalis recoverable from the lesions, and the phase of recovery from PIA. The findings in this litter will be considered in greater detail, as this is the first time that the condition has been followed sequentially.

From the pigs killed at twenty three days and forty days post-dosing mucosalis was recovered, but mostly in numbers lower than those isolated from affected animals in the field, and evidence of PIA was seen only in the L.I. of the second of these pigs. At this stage the bacteria could not be demonstrated by electron microscopy or silver-staining, probably due to the small number present, although the presence of mucosalis antigen was confirmed intracellularly in the immunofluorescence studies. These pigs could possibly be considered to be in the pre-adenomatous period of the condition, in which there was poor growth rate, no definite evidence of PIA, but with mucosalis established in the alimentary tract.

In the pigs killed at fifty days and fifty two days post-dosing, there was evidence of PIA and NE. Mucosalis was recovered and its presence intracellularly confirmed by silver-staining,

immunofluorescence, and electron microscopy. The relationship between mucosalis and the epithelial cell was comparable to existing descriptions of this stage of the condition (Rowland and Lawson, 1974; Chapter III, Part A). There was clinical evidence of poor growth rate and wasting, gross and/or histological evidence of PIA, mucosalis could be demonstrated within the affected epithelial cells and recovered in large numbers from the lesions.

In the other pigs from this litter there was evidence of recovery from the condition. In the two pigs killed at fifty eight days post-dosing there was gross and histological evidence of PIA. Mucosalis was not recovered from one of these pigs (183/76) and was only recovered in reduced numbers from the other (189/76). There was histological, immunofluorescence and ultrastructural evidence that these pigs were at an early stage of the recovery phase of PIA. Evidence is presented in Chapter IX that these bacteria may have had antibody attached to their surface at this stage, which might in part explain the failure to isolate mucosalis or its recovery in reduced numbers. The three pigs killed at sixty four and sixty five days post-dosing mostly showed only slight or no gross evidence of PIA, although adenomatous change was present at the histological level. Mucosalis was not recovered from these pigs, but its presence was demonstrated by silver-staining, immunofluorescence and electron microscopy. As with the pigs killed at fifty eight days post-dosing there was evidence that these three pigs were in the recovery phase of PIA although at a later stage. In these three there was resolution of the lesions, so that only

histological evidence of adenomatous change was present, and the pigs had clinically begun to show improved appetites and weight gains. The failure to recover mucosalis can possibly again be attributed to antibody coating of the bacteria (Chapter IX). The macrophages containing bacteria resembling mucosalis seen in the lamina propria by immunofluorescence and electron microscopy, and the neutrophils and macrophages with identical bacteria seen in the gland lumina on electron microscopy are also indicative of the recovery phase of PIA. Although recovery of pigs with PIA has been suggested previously on clinical grounds no study of the regression of the lesions has been reported. The recovery phase of PIA is considered in greater detail in Chapter IX.

The pathological findings in the L.I. of pig 140/76 from the litter of sow G are of interest with respect to the suggested development of NE from PIA (Rowland and Lawson, 1975b). In this pig in the areas of adenomatous change in the L.I. there was extensive necrosis with a large number of balantidia present on the surface. The adjacent areas of mucosa, with a large goblet cell population, were relatively free of balantidia. The immaturity of the adenomatous epithelium appeared to make it highly susceptible to the microbial flora of the L.I., with resultant tissue destruction. Further evidence for this relationship between PIA and NE postulated by Rowland and Lawson (1975b) is provided by the occurrence of both conditions in the litter of piglets (sow G), which were dosed with mucosalis, and the mucosa from a case of PIA.

In these experiments the reproduction of PIA and NE in

neonatal piglets was achieved. It is realised that these experiments were not fully controlled, but under the experimental conditions pertaining at this stage of the investigation this was not thought to be possible. However, a number of factors are indicative, These are:

- (i) the failure to induce comparable changes in six litters of piglets exposed to mucosalis alone (Chapter IV).
- (ii) the appearance of adenomatosis in a high percentage of the piglets exposed to mucosa and mucosalis.
- (iii) the appearance of the disease at a different age to that at which it normally occurs.
- (iv) the absence of mucosalis and intestinal changes in pigs sacrificed at the start of the experiment, suggest that the condition has been successfully transmitted, albeit that this result needs confirmation.

There is a real problem in obtaining pigs for experimental purposes from a "clean" source, of which it can be stated categorically that PIA does not occur, and that mucosalis is not present within animals in the herd. One could also argue that it is better to use animals from a herd in which the animals are known to be capable of contracting the disease, rather than animals from a herd in which the condition is thought never to occur, as these latter animals may be resistant to mucosalis infection rather than never exposed. In these experiments the sows were obtained from herds in which PIA is known to occur and the piglets from these sows in isolation were used to reproduce the condition.

Mucosalis strains from different pigs show detectable differences in their surface antigenicity, although the majority share some of these factors (Lawson, Rowland and Roberts, 1977b). A number of the mucosalis strains isolated from the experimental piglets of the litter of sow G were examined by Dr. G.H.K. Lawson (Department of Veterinary Pathology, Edinburgh University Veterinary Field Station) and their surface antigenic factors compared to those of mucosalis strain 106/75 and strains recovered from the adenomatous mucosa used to dose the piglets of sow G. They more closely resembled those isolated from the adenomatous mucosa used for infection than the strain 106/75. This might suggest that the mucosalis organisms present in the adenomatous mucosa were those establishing in the experimental animals with the resultant development of PIA, although other explanations for this observation are possible.

At the present time it is not known why in the neonatal piglet model adenomatous mucosa is successful in transmission of the condition, whilst exposure to the organism alone is not. Possible factors include the loss of virulence of mucosalis on culture on laboratory media, other factors in the mucosa may be necessary or other organisms in the mucosa may be essential. Other organisms which could be differentiated from mucosalis were observed in the lesions of NE of the experimental animals. In this respect the observed disease appears similar to proliferative ileitis in the hamster. Although the latter condition has been transmitted using affected tissues, transmission using cultures of single bacterial

species recovered from the lesions has been unsuccessful (Jacoby et al., 1975; Amend et al., 1976). However, Jacoby et al. (1975) found that mixed bacterial flora isolated from ileal lesions was capable of transmitting the condition, but the morbidity and mortality were reduced compared to transmission obtained using ileal extracts. In the hamster, however, the evidence that the organism present within the affected epithelial cells is the organism isolated in culture is more tenuous than has been presented in PIA.

Recently, in a study of the pathogenicity of Neisseria gonorrhoeae (Novotny, Short and Walker, 1975; Novotny et al., 1977), "infectious units" have been described. These were collections of gonococci apparently multiplying in special clusters, and the evidence suggested that they were formed inside phagocytic cells and had a special affinity for epithelial cells. An infectious unit was defined as a cluster of multiplying gonococci surrounded by granules and remnants of phagocytic cells, and it was suggested that their formation was an expression of the pathogenicity of gonococci. Serial sections showed that the infectious units still held the shape of the cells in which they were formed, and around each cluster of cocci was the partially preserved remnant of the phagocyte membrane. The granules surrounding the cocci were of human cell origin and contained typical host cell organelles such as mitochondria and nuclear remnants. The infectious units were thought to be formed intracellularly in macrophages, and that the accumulation of granules around the gonococci was an active process and not just simple physico-chemical absorption. The

contact of gonococci, in infectious units, with epithelial cells was mediated through the granules surrounding them, and most infectious units were seen in close contact with epithelial cells in urethral pus. Previously greater attention had been paid to the interaction between single gonococci and human cells (Swanson, 1973; Witt, Veale and Smith, 1976) and the role of pili in this attachment. Although a direct analogy between mucosalis and this work on gonococci can obviously not be made it may be that mucosalis in the epithelial cell or newly released from the cell may possess properties that increase its infectivity for the porcine intestinal epithelium when compared to mucosalis grown on artificial media. It is interesting that in smears of faeces and impression smears of mucosa from affected animals mucosalis is seen intracellularly and free, either singly or in clumps.

The adenomatous mucosa which had been used when fresh to dose the piglets of sow G was also, after storage at -80°C for fifty one days, used to dose the piglets of sow H. In this latter litter however, only one piglet had evidence of PIA. Whether this is an indication that infectivity is lost on storage at -80°C cannot be concluded from this one experiment. The numbers of mucosalis recovered from this mucosa, after storage at -80°C , were almost identical to the numbers recovered from the fresh mucosa. With B. piliformis, Craigie (1966a) described a reduction of 99% or greater in the infectivity of yolk sac suspensions stored at -75°C . This is measured as a reduction of infectivity for embryonated hen's eggs, and since B. piliformis was not grown on artificial laboratory media

it is not known whether this would have shown a similar reduction. Schaich Fries (1977) found that yolk sac materials infected with B. piliformis were still infective for mice and embryonated eggs after storage at -80°C for twenty four days, but not after fifty two days. Ganaway, Allen and Moore (1971) however, found that yolk sac suspensions were infective after storage at -70°C for four and a half years. This difference may be related to the numbers of spores present. Sporulation does not occur in mucosalis. It is possible that mucosalis in the stored mucosa was less able to survive in the intestinal tract of the pig, and enter the epithelial cells, although still able to grow on laboratory media.

With the litter of sow F although there was some success in transmission of the condition this was less than for the litter of sow G. Whether the fact that these piglets had received the non-adenomatous large intestinal mucosa from pig 71/76 first was of significance is unknown. The presence of the bacterial flora from this sample may have meant that the mucosalis in the adenomatous mucosa was unable to compete successfully in the environment of the intestinal tract. Another possibility is that damage to the epithelium prevented the entry/uptake of mucosalis into the epithelium. Since the factors surrounding this stage of infection are unknown it is not possible to evaluate what effect these factors might have had.

Mucosalis was not recovered from the oral cavity of any of these piglets in any of the three litters. This is in contrast to the experiments described in Chapters IV and VI. The mucosal

suspension for dosing was more viscid and after administration by short intra-gastric tube regurgitation was less common than with a suspension of mucosalis. However, most of the piglets also received a mucosalis suspension in addition to the mucosa.

The pathological and histopathological appearance of piglets 108/76 and 123/76 resembled that described in Chapter III, Part D, for the "three weeks scours" syndrome. The significance of this is not clear, but adds weight to the suggestion that the role of mucosalis in this type of condition should be considered.

Pig 157/76 (litter of sow G) is of interest. It had lost weight, but there were no gross lesions of PIA at necropsy. Mucosalis was not recovered from this case, but histologically there were isolated adenomatous glands present in the mucosa. Mucosalis was probably not recovered from this pig due to few, if any, adenomatous glands being present in the area of mucosa sampled bacteriologically. The difficulty of reaching a diagnosis in a pig presenting these features can be easily realised and the value of histological examination in such circumstances is obvious.

The recovery of mucosalis from pig 219/76 is worthy of further consideration. This piglet was killed at seventy six days old, after dosing with non-adenomatous mucosa at birth and with adenomatous mucosa at twenty four hours of age. In the infectivity experiments mucosalis was recovered up to forty days post-dosing in neonatal pigs (Chapter IV). In the litter of sow G, mucosalis was recovered up to fifty eight days post-dosing, after which time recovery of mucosalis was not made from the pigs in the litter and the

lesions resolved. The isolation of mucosalis, at seventy five days post-dosing with adenomatous mucosa, from pig 219/76 would not seem to be in accordance with these results. This pig was, however, mixed with the pigs of the litter of sow G from forty seven days of age, and all these animals had lesions of PIA and were probably excreting mucosalis in the faeces. It seems more likely that infection was from this source, and the time from contact with the affected pigs to euthanasia and recovery of mucosalis was twenty nine days.

In summary, further evidence for the infective nature of PIA is presented, and information on the incubation period is provided. The neonatal piglet would seem to provide a suitable model for investigation of the condition, if the results can be repeated consistently. A sequential study of the development and regression of the condition was possible.

CHAPTER VIII

TRANSMISSION EXPERIMENTS USING POST-WEANED PIGSINTRODUCTION

Although the infectivity experiments (Chapters IV, V and VI) showed that the neonatal piglet was more susceptible to mucosalis infection after oral dosing with cultures of mucosalis than the post-weaned pig, the condition in the field is seen in the latter age group of animals. Pharmacologically induced hypomotility using benzetimide was shown to increase susceptibility to mucosalis in the post-weaned pig.

Evidence has been published suggesting that infection with mucosalis can be prevented by oral medication with an antibiotic feed supplement containing chlortetracycline (100 g per tonne), penicillin (50 g per tonne) and sulphadimidine (100 g per tonne) (Love, Love and Edwards, 1977; Love and Love, 1977). An attempt was made using this information to rear susceptible piglets by feeding an antibiotic supplement. Initially the sow was treated parenterally, then by adding antibiotics to the feed and later the piglets creep was medicated with antibiotics. Such piglets should be fully susceptible to mucosalis infection (Love and Love, 1977). These piglets were then challenged with mucosa from an animal with PIA, or a suspension of mucosalis, after pharmacologically induced hypomotility had been effected.

MATERIALS AND METHODS

Infecting organism

The strain of mucosalis 106/75 SI 10^{-3} was used in these experiments. Those piglets, which were orally dosed with a suspension of mucosalis, received 2×10^{10} viable mucosalis.

Source of Mucosa used for dosing

The mucosa used in this experiment was derived from a confirmed case of PIA, pig 1152/76 (Chapter IX). The estimated number of viable mucosalis per gram of mucosa is given in Chapter IX, Table 9a. The suspension of mucosa used for dosing was prepared as described in Chapter II. Those piglets which received mucosa, were dosed with 10 mls of the homogenised suspension. The suspension contained equal volumes of mucosa and tryptose phosphate broth. Although mucosalis was recovered in large numbers from this pig, histological, immunofluorescence and electron microscopic examinations suggested that it may have been in the early recovery phase of PIA (see Chapter IX) when the mucosa was harvested.

Benzetimide

This was administered orally at a dose of 0.250 mgs per kg body weight. The required volume of a 0.25% solution was made up to approximately 10 mls in S.D.W.

Experimental animals and timing of procedures

The piglets used in this experiment were born to two sows, K and L, from farm A. These sows farrowed three days apart, sow K

producing thirteen piglets, one of which was crushed, and sow L producing nine piglets. Sow K received oxytetracycline (Engemycin) by intra-muscular injection from five days before farrowing to ten days after the birth of her litter. Sow L received similar treatment for eight days and seven days respectively. From this time both sows received meal with 'Cyfac 25' added. The sows were fed 'A.B.R.O. Breeders' meal (Seafield Mill, Roslin). From seven days and four days post-partum, respectively, the litters of sows K and L were offered 'starter creep' (Seafield Mill, Roslin) medicated with 'Cyfac 25', at the level indicated in Chapter II. The amount of creep offered to the piglets was increased as they grew. The piglets were weaned at eighteen days (litter, sow L) and twenty one days (litter, sow K) of age. From this time they received decreasing quantities of creep feed and increasing amounts of 'A.B.R.O. Breeders' meal. Food and water were offered ad-lib and the food had been medicated with 'Cyfac 25'.

Four days before oral dosing with mucosa and mucosalis, medication of the feed with 'Cyfac 25' was stopped. Twenty four hours before infection the piglets were weighed and divided into three groups, so that each group was made up of three piglets from the litter of sow L and four piglets from the litter of sow K. This selection was random. At this time each of the twenty one piglets was dosed orally with benzetimide. Each of the three groups of seven piglets were put into separate rooms without contact and precautions taken to prevent spread of any infection from one group to another. Piglets were forty two to forty five days old when

infected, and one group (seven piglets) received benzetimide, chalk suspension and mucosalis, the other benzetimide, chalk suspension, mucosalis and mucosa, with the control group receiving benzetimide alone. The piglets were weighed weekly, and pigs from each group were killed at intervals from four weeks to seven weeks post exposure.

Sites examined

Tissues from the M.S.I., T.S.I. and L.I. of all these piglets were examined bacteriologically and material was fixed for histology, immunofluorescence and electron microscopy. Mouth swabs were not examined from these piglets.

Bacteriological examination

Modified Z.-N. stained impression smears from the M.S.I., T.S.I. and L.I. were examined. Mucosal samples from these sites were inoculated onto B.A. and N.B.G. at the $\frac{1}{20}$, $(\frac{1}{20})^2$, $(\frac{1}{20})^3$ and $(\frac{1}{20})^4$ dilutions.

Calculation of CUSUM values

The figures for body weight were treated as described in Chapter III, Part C.

$$\text{CUSUM} = \text{gain in body weight} - 2.0$$

As in Chapter VII, the arbitrarily chosen constant 2.0 was employed.

RESULTS

Clinical Findings/Daily Observations

Both sows and piglets remained healthy throughout the suckling period and the piglets were eating medicated creep in reasonable quantities by the time they were weaned. After weaning, the piglets readily ate meal and there was no evidence of diarrhoea. For the ten to fourteen days after oral dosing the group which had received both mucosa and mucosalis consumed less meal and appeared to eat less greedily than the other two groups. After this time, however, although there was still more food left by this group they showed increasing interest when fresh meal was offered. All three groups of piglets were fed ad-lib and uneaten food was removed after 24 hours, and replaced. Throughout the experiment no clinical evidence of any disease was observed. Pigs were weighed weekly from the day before oral dosing and the weekly weights and the CUSUM values calculated from these figures are presented in Tables 8a, 8b and 8c. Pigs for euthanasia were selected on these CUSUM values, the poorest pigs being chosen each time. From these tables it can be seen that in the two weeks after dosing the piglets exposed to mucosa and mucosalis grew less well than the piglets in the other two groups.

Pathological Results

Gross Pathology/Histopathology

The pathological findings in the three groups of piglets are presented in Tables 8d, 8e and 8f. There were no gross lesions detected in the carcasses of any of the piglets from the three groups

Table 8a. Body Weights and CUSUM Values for the Control Group

Sow	Pig Reference	Age														
		6 wks		7 wks		8 wks		9 wks		10 wks		11 wks		13 wks		
		W	C	W	C	W	C	W	C	W	C	W	C	W	C	
L	436/76	16.00		17.50	-0.50	21.00	1.00	22.00	0							
L	446/76	16.00		19.00	1.00	20.00	0	24.00	2.00	26.00	2.00					
K	447/76	13.50		16.00	0.50	19.00	1.50	22.00	2.50	24.00	2.50					
K	470/76	16.50		19.50	1.00	23.00	2.50	26.00	3.50	30.00	5.50	34.00	7.50			
L	471/76	16.00		19.00	1.00	21.00	1.00	24.00	2.00	29.00	5.00	32.00	6.00			
K	490/76	21.00		24.00	1.00	28.00	3.00	32.00	5.00	36.00	7.00	40.00	9.00	47.00	12.00	
K	491/76	15.50		19.00	1.50	21.00	1.50	24.00	2.50	26.50	3.00	34.00	8.50	35.50	6.00	

W = Body weight in lbs.

C = CUSUM value.

At 13 weeks there had been an interval of 2 weeks since the previous weighing and CUSUM = gain in body weight - 4.0.

Table 8b Body Weights and CUSUM Values for Piglets Infected with Mucosalis only.

Sow	Pig Reference	Age															
		6 wks		7 wks		8 wks		9 wks		10 wks		11 wks		13 wks			
		W	C	W	C	W	C	W	C	W	C	W	C	W	C		
L	438/76	11.00		11.50	-1.50	14.00	-1.00	17.00	0								
K	453/76	16.00		19.00	1.00	21.50	1.50	26.00	4.00	27.00	3.00						
K	454/76	10.00		12.00	0	14.50	0.50	18.00	2.00	20.00	2.00						
L	480/76	17.00		20.00	1.00	24.00	3.00	30.00	7.00	30.00	5.00	35.00	8.00				
L	481/76	16.00		18.00	0	22.50	2.50	26.00	4.00	30.00	6.00	34.00	8.00				
K	498/76	17.50		22.00	2.50	27.00	5.50	33.00	9.50	36.00	10.50	44.00	16.50	56.00	24.50		
K	499/76	18.00		22.00	2.00	26.50	4.50	31.00	7.00	34.00	8.00	37.00	9.00	46.00	14.00		

W = Body weight in lbs.

C = CUSUM value.

At 13 weeks there had been an interval of 2 weeks since the previous weighing and CUSUM = gain in body weight - 4.0.

Table 8c Body Weights and CUSUM Values for the Piglets Exposed to Mucosa and Mucosalis.

Sow	Pig Reference	Age															
		6 wks		7 wks		8 wks		9 wks		10 wks		11 wks		13 wks			
		W	C	W	C	W	C	W	C	W	C	W	C	W	C		
L	439/76	11.50		11.50	-2.00	12.00	-3.50	14.00	-3.50								
K	455/76	8.00		9.00	-1.00	9.50	-2.50	12.00	-2.00	14.00	-2.00						
L	464/76	16.00		17.00	-1.00	19.00	-1.00	22.50	0.50	24.00	0						
K	482/76	14.50		15.50	-1.00	18.00	-0.50	20.00	-0.50	23.00	0.50	26.00	1.50				
L	487/76	12.00		13.00	-1.00	15.00	-1.00	18.00	0	20.00	0	26.00	4.00				
K	500/76	13.50		15.00	-0.50	18.00	0.50	20.00	0.50	22.00	0.50	25.00	1.50	35.00	7.50		
K	503/76	18.00		21.00	1.00	24.00	2.00	28.00	4.00	32.00	6.00	39.00	11.00	50.00	18.00		

W = Body weight in lbs.

C = CUSUM value.

At 13 weeks there had been an interval of 2 weeks since the previous weighing and CUSUM = gain in body weight - 4.0.

Table 8d Pathological Results - Control Group

Pig Reference	Day Necropsied (Post-dosing)	Gross and Histopathological Findings	
436/76	26 days	M.S.I.	A number of crypts contain neutrophils
446/76	32 days	N.A.D.	
447/76	32 days	L.I.	Some oedema of the lamina propria, and detachment of the surface epithelium.
470/76	39 days	N.A.D.	
471/76	39 days	N.A.D.	
490/76	46 days	L.I.	surface damage
491/76	46 days	L.I.	surface damage

N.A.D. = No abnormality detected.

Table 8e Pathological Results - Mucosalis only Group

Pig Reference	Day Necropsied (Post-dosing)	Gross and Histopathological Findings	
438/76	27 days	N.A.D.	
453/76	34 days	L.I.	Surface epithelial damage, the epithelium is flattened, with bacteria closely applied to the surface.
454/76	34 days	As 453/76	
480/76	41 days	L.I.	Slight damage to surface epithelium, balantidia occasionally seen.
481/76	41 days	As 480/76	
498/76	48 days	L.I.	Slight surface damage.
499/76	48 days	L.I.	In some areas, surface epithelium is cuboidal rather than columnar.

N.A.D. = No abnormality detected.

Table 8f Pathological Results - Mucosa and Mucosalis Group

Pig Reference	Day Necropsied (Post-dosing)	Gross and Histopathological Findings
439/76	27 days	M.S.I., T.S.I. - crypts contain neutrophils L.I. - grossly the mucosa was marginally thickened. The crypts are elongated and crowded, and there is surface epithelial damage with bacteria applied to the surface. Increase in cells in the lamina propria.
455/76	35 days	L.I. - surface epithelial damage, with bacteria adherent to the surface.
464/76	36 days	L.I. - As 455/76, with balantidia also present.
482/76	42 days	M.S.I. Crypts have a crowded appearance, and neutrophils in lumina. L.I. - surface damage.
487/76	43 days	L.I. - surface damage.
500/76	49 days	M.S.I., T.S.I. - Very elongated crypts with a crowded appearance, Increase in cells in the lamina propria.
503/76	50 days	M.S.I. Occasional crypts contain cell debris.

except that in pig 439/76 the mucosa of the L.I. appeared slightly thickened.

Microscopically there was no evidence of PIA in any of the pigs although in the pigs killed at twenty seven and forty two days from the mucosa and mucosalis group, a number of crypts in the small intestine contained neutrophils and cell debris. This was not seen in the control or mucosalis only group to the same extent, if at all. The thickening of the L.I. of pig 439/76 was confirmed histologically, but changes of PIA were not present. A number of the piglets from all three groups showed some surface damage in the large intestine, with bacteria including vibrios closely adherent to the surface epithelium. In silver-stained sections no intracellular bacteria were seen within the epithelium.

Electron Microscopy

Electron microscopic examination was not carried out on tissues from these piglets.

Bacteriological Results

Examination of modified Z.-N. stained impression smears of the mucosa of the M.S.I., T.S.I. and L.I. of all these pigs failed to demonstrate acid-fast vibrios either free or in an intracellular site. Mucosalis was not recovered from any of these animals.

Immunofluorescence Results

Tissues from the M.S.I., T.S.I. and L.I. of all these pigs with one exception (439/76) were negative when stained with mucosalis anti-serum, and F.I.T.C. conjugated G.A.R. In pig 439/76 in the

lamina propria of the M.S.I. and T.S.I. there were occasional positive fluorescing cells. The antigen in these cells was arranged around the periphery, and the cells were probably macrophages or other cells of the phagocytic series.

DISCUSSION

In this experiment there was no evidence that PIA had been reproduced in any of the piglets. Neither was there any evidence of the establishment of infection with mucosalis, except for the presence of cells in the lamina propria of the small intestine of the pig killed at twenty seven days post-dosing. Antigen is retained in macrophages for some time after uptake (Unanue and Askonas, 1968), and possibly this antigen was taken up into the mucosa after the oral dosing with mucosa and mucosalis. An alternative explanation is that infection with mucosalis had been established, recovery had occurred with regression of any lesions present and mucosalis antigen was still retained within the cytoplasm of macrophages in the lamina propria.

The group of piglets which received mucosa and mucosalis on clinical assessment and CUSUM values grew less well and had poorer appetites than the piglets in the other two groups. There is no evidence that this was due to mucosalis infection or to PIA. The other microbial flora in the mucosa may have been responsible, and in support of this were the other minor inflammatory lesions which were present, especially in the L.I. of a number of the pigs. The mucosa may have been responsible through other mechanisms for the poorer performance of this group.

The reasons for the failure to transmit the condition in this experiment are not fully understood, although it seems pertinent to discuss a few possible factors which may have played a part. Neonatal piglets were found to be more susceptible to infection with mucosalis when dosed orally with pure bacterial cultures and a similar situation may pertain with respect to mucosalis in adenomatous mucosa. Consideration of the number of viable mucosalis in the mucosa or the weight of mucosa used for infection in relation to the weight of the animals exposed, show that the dose received by the post-weaned pigs in this experiment is considerably lower than that received by the neonatal piglets in the previous successful experiments. The adenomatous mucosa used for dosing yielded large numbers of mucosalis on culture, but on histological, immunofluorescence and ultrastructural examination there was evidence that the pig was at the early stage of the recovery phase of PIA. As discussed in Chapter IX, antibodies to mucosalis may be involved in this stage of the condition. So, although mucosalis was recovered on artificial media from the mucosa, there may have been some change in the bacteria or sufficient antibodies present to reduce the infectivity in vivo.

The apparent lack of effect of benzetimide is in contrast to the situation described in Chapter V. These pigs were killed from four weeks post-dosing in contrast to those in Chapter V, which were killed at approximately fourteen days post-dosing. The possibility exists that these piglets were not susceptible to mucosalis infection, as with sow C in Chapter IV, where the possibility of individual susceptibility is discussed.

The feeding of 'Cyfac 25' should have prevented possible infection by mucosalis, and so the piglets should have been fully susceptible to mucosalis at the time of exposure. Most strains of mucosalis derived from U.K. sources have a minimal inhibitory concentration to tetracycline of 0.5 μ gs/ml or less and from in vitro tests the tetracycline would appear to be the agent likely to be active in 'Cyfac 25' (Dr. G.H.K. Lawson, personal communication, 1977). Circumstances for the establishment of mucosalis and development of PIA may not have been correct and it is possible that factors, in addition to peristalsis, have to be overcome. Further work will be necessary to clarify these points, but at present concentration on the neonatal piglet in experimental work would seem to be justified.

In summary successful transmission of PIA was not achieved in the post-weaned pig in contrast to the situation in the neonatal pig. Possible reasons for this failure are discussed.

CHAPTER IX

STUDIES ON THE RECOVERY PHASE OF PIAINTRODUCTION

Clinical observations suggest that ^amay pigs affected with PIA recover after a period of wasting, progress to slaughter thereafter is unremarkable, and no gross lesions are visible in the alimentary tract at this time (Rowland and Rowntree, 1972; Rowland and Lawson, 1974). The study reported in Chapter III, Part C, on a closed M.D. herd supports this opinion. A number of pigs grew poorly, those sacrificed proved often to have lesions of PIA and the others, after a number of weeks began to grow more rapidly and proceeded to slaughter weight without further setback.

That pigs which have had PIA produce a serological response to the intracellular bacteria was shown by Rowland and Lawson (1974). They demonstrated that F.I.T.C. conjugated serum from affected animals reacted with antigen in the apical cytoplasm of adenomatous epithelial cells. Such circulating antibody is likely, however, only to be one component of the immune response and clearly gut associated immunoglobulin could be much more important in the immunity evoked in response to an agent apparently largely confined to this tissue. Secretory immunity has been described operating in the intestinal tract, and secretory immunoglobulins (IgA and IgM) are transported from plasma cells in the lamina propria through the crypt epithelial cells onto the mucosal surface (Brandtzaeg and Baklien, 1976). A preliminary study of the possible significance of secretory immunoglobulins in the recovery from PIA was carried out on the animals studied in this chapter.

In the transmission experiments (Chapter VII), sequential killing of affected animals indicated that recovery took place with the elimination of mucosalis infection and regression of the adenomatous lesions. These animals formed a basis for the description of the changes that take place during recovery and for the recognition of the recovery phase in naturally occurring cases of PIA.

The recovery from this condition is of great interest due to the neoplastic character of the lesion and the constant association with intracellular bacteria. Extensive investigation on immunity to intracellular bacteria and protozoa has been undertaken, but mostly this work has been carried out on organisms that parasitise cells of the mononuclear phagocytic series (Hahn, 1975), and no comparative information is available on the development of immunity in intracellular bacterial infection of epithelial cells.

MATERIALS AND METHODS

Animals

The animals used in this study included naturally occurring cases of PIA, and the experimental cases described in Chapter VII. Control material was obtained from the typical PIA cases¹, and the control pigs, with no evidence of intestinal abnormality, described in Chapter III, Part A.

¹ Typical PIA is used to describe those cases of PIA from which mucosalis was recovered in large numbers, and evidence of recovery as described in this chapter was not present. The cases described in Chapter III, Part A, conform to this description.

Pathological and bacteriological examination

Tissue for histological, electron microscopic and bacteriological examination was taken and processed as described in Chapter II.

Immunofluorescent examination

Cryostat sections were stained in a sandwich test using mucosalis rabbit antisera and F.I.T.C. conjugated G.A.R. For this either 1248/72 2C2 OH mucosalis anti-sera or 982/76 mucosalis anti-sera was used. In addition sections were stained with F.I.T.C. conjugated rabbit anti-pig γ -globulin. This latter was obtained from Dr. G.H.K. Lawson (Department of Veterinary Pathology, Edinburgh University Veterinary Field Station) and details of preparation were given in Chapter III, Part C.

RESULTS

Pathological Results

Animals in the recovery phase of PIA were compared with the pigs showing lesions typical of PIA, and with pigs free of alimentary tract abnormalities, and since both these groups were described fully in Chapter III, Part A, they will not be considered again here.

It is thought that it might help the reader to follow the progression of the condition if division is made into early, intermediate and late stages of recovery. It is stressed, however, that this is a dynamic process and that such clear-cut separation cannot therefore necessarily be made in all cases.

Gross Findings

At necropsy most pigs in the early stage of recovery had visible lesions of PIA (Rowland and Rowntree, 1972; Rowland and Lawson, 1974; Chapter III, Part A). There was thickening and exaggerated folding of the mucosa of variable lengths of the small and large intestines, indistinguishable from that described in typical PIA. In the later stages the alimentary tract usually appeared normal to the naked eye, but occasionally the mucosa was marginally thickened.

Histopathology

The early stage of the recovery phase

At this period the mucosa was thickened and adenomatous and the surface was flattened and lacked villi; in which features it did not differ from typical PIA. However, in the more basally situated glands there were a variable number of goblet cells, not usually more than two or three per gland. There was an increased cell loss from the epithelium seen as clumps of cells being extruded from the surface. In comparison with typical PIA cases there was an increase in the amount of cell debris in the lumina of glands, this consisted of desquamated epithelial cells and also neutrophils.

The intermediate stage of the recovery phase

More goblet cells were seen in the adenomatous glands, and occasionally glands were seen which were enlarged and appeared hyperplastic, but in which almost all or all of the cells were mucus-secreting cells (Figure 76). At this time there was attempted villus

formation so that short villi were seen on the surface, and often the epithelium over these villi was continuous with that of an underlying adenomatous gland. The epithelium on the villi was usually adenomatous but towards the villus tip it became more normal in appearance, i.e. simple columnar (Figure 76). Associated with the recovery and attempted villus formation microscopic "adenomas" were observed (Figure 77). These consisted of a number of adenomatous glands overlain by more normal epithelium and the whole "adenoma" was raised above the surrounding mucosa, which was mostly normal in appearance.

During the recovery phase of PIA neutrophil polymorphs were sometimes seen within the lumina and epithelium of adenomatous glands, and in the surrounding lamina propria. Less often eosinophil polymorphs were seen in large numbers in these situations. Although seen at all stages of the recovery phase the infiltration of neutrophils was most prevalent during the intermediate stage.

The late stage of the recovery phase

As recovery proceeded the mucosa was no longer increased in width and those adenomatous glands present were near to the surface of the mucosa, and appeared to be "growing out" (Figure 78). The underlying mucosa was normal, the glands were smaller, lined by simple columnar epithelium and had a variable goblet cell population. During the later stages of the recovery phase there were only occasional isolated adenomatous glands present within the mucosa. A variable number of goblet cells were present in these glands. In the small intestine the remaining mucosa was relatively flattened and showed

severe or total villous atrophy, with associated crypt lengthening and high mitotic activity. In the large intestine the crypts were lengthened and hyperplastic in the mucosa surrounding the isolated adenomatous glands.

There was an increase in the cells in the lamina propria during the recovery phase. The number of infiltrating cells varied with the stage of recovery, the increase being less marked early in the process but substantial at the late stage of the recovery phase. The numbers of lymphocytes, plasma cells and macrophages were all increased. Macrophages were also seen within the epithelium and lumina of the glands especially in the intermediate and late stages (Figure 79).

Deeply eosinophilic inclusions were seen in the epithelium predominantly (Figures 80 and 81), but also occasionally in the lamina propria immediately adjacent to the basement membranes of the adenomatous glands, and less often in the lumina of glands. These inclusions varied in size from approximately one twentieth to one quarter the size of the epithelial cell nucleus, and were more or less spherical in shape. Mostly they were situated basally in the epithelial cells. They were not seen in all glands, some had only occasional inclusions in a small number of cells, whereas in others many cells had inclusions and there were several in each cell. In Feulgen stained sections these inclusions stained Feulgen positive, indicating the presence of D.N.A. In silver-stained sections some of these inclusions stained (Figure 83). The inclusions were mostly a feature of the intermediate and late stages of recovery.

In silver-stained sections bacteria were seen in the apical cytoplasm of affected intestinal epithelial cells (Figures 82 and 83). These bacteria were often arranged in aggregations or clumps, although single bacteria of vibrio morphology were also seen. The bacteria were not restricted to the apical cytoplasm, although lying predominantly in that site. Aggregations of bacteria were also seen nearer to the base of the cell. There were cells present in the lumina of glands, possibly macrophages, neutrophils or desquamated epithelial cells, which also contained argyrophilic vibrio-shaped bacteria (Figures 82 and 83). Non-epithelial cells containing identical bacteria were also seen within the adenomatous epithelium and in the surrounding lamina propria, and were most likely to be macrophages. Those in the lamina propria were in close proximity to the basement membranes of the glands. In areas where the adenomatous glands were growing out, bacteria could be demonstrated within the superficial, adenomatous glands but not in the underlying histologically normal glands (Figure 84). Where only isolated adenomatous glands were present most of the cells in these glands contained argyrophilic vibrios (Figure 85). In these adenomatous glands the mucus-secreting cells did not contain vibrios. The epithelium of the surrounding histologically normal glands was free of bacteria (Figure 85). However, cells in the lamina propria containing bacteria were seen in close proximity to both the adenomatous glands and the normal glands, without bacteria in the epithelium (Figure 85).

Electron Microscopy

The early stage of the recovery phase

Cases in the recovery phase of PIA did not superficially differ greatly from the ultrastructural appearance of typical PIA as previously described. There were curved bacteria free within the apical cytoplasm of the affected intestinal epithelial cells, which cells had the ultrastructural features of immature crypt cells. In some cases there was evidence of epithelial damage so that there were cytoplasmic blebs formed at the luminal surface of the epithelial cells. It appeared as if these blebs of cytoplasm were being extruded into the lumina of the glands. Occasionally disruption of the plasma membrane with loss of cytoplasmic contents was seen. A number of the mitochondria were swollen, with disintegrating cristae and the formation of matrix granules. Where degenerative changes were present, not all the epithelial cells were involved. Less often at this stage bacteria were seen within phagolysosomes in the intestinal epithelial cells, or were membrane-bound (Figure 86), and showing evidence of degenerative change. Occasional bacteria free in the cytoplasm were shrunken and more electron dense. A less prominent feature at this stage was the presence of bacteria within phagosomes or phagolysosomes in macrophages in the lamina propria. Another variable feature was the presence of cell debris and neutrophils in the lumina of glands, but generally this was more common at later stages.

The intermediate stage of the recovery phase

When recovery was more advanced many of the bacteria free within the cytoplasm of the epithelial cells were more electron-dense

and shrunken in appearance (Figures 87 and 88). Their usual features in this case were less clear and the cell wall and plasma membrane were not clearly differentiated. The bacteria were mostly present within the apical cytoplasm and seemed to form collections or aggregations in that site. They were less restricted to that area than in the typical PIA cases already described, and bacteria were often seen deeper in the cell. Bacteria were also occasionally seen in which the cell wall was apparently incomplete and sections of the wall were thickened and extremely electron dense (Figure 89). Bacteria singly (Figure 86) or in larger numbers (Figures 90 and 91) were seen in membrane-bound vesicles. The bacteria present within these vesicles often showed degenerative changes. The plasma membrane and cell wall were distinct and separated. The cytoplasm had electron translucent areas, was clumped and often retracted away from the surrounding plasma membrane and cell wall. The phagosomes or phagolysosomes containing bacteria were of two types; some were large and contained few bacteria in a large space (Figure 92), others varied in size but were packed full of organisms (Figures 90 and 91). At the base of some epithelial cells there were phagolysosomes containing cell debris, in which the outlines of bacteria could sometimes be recognised. Within the epithelial cells more complex phagolysosomes resembling autophagolysosomes (Cheville, 1976) (Figure 93), and apoptotic bodies (Kerr, Wyllie and Currie, 1972) (Figure 94) were also seen. Those Feulgen positive inclusions described histologically corresponded to these apoptotic bodies. They were mostly situated basally in the epithelial cells (Figure 94), but were also seen above the nucleus. As well as in the epithelium they occurred

in the lamina propria, adjacent to the gland basement membrane, in cells resembling macrophages (Figures 95, 102 and 103). They were also seen apparently passing from the epithelium to the lamina propria (Figures 96 and 97). In the glands' lumina they occurred both free and within macrophages (Figure 98).

Bacteria were also seen within phagosomes or phagolysosomes in phagocytic cells, resembling macrophages, in the lamina propria (Figure 99). The cells conformed to the published descriptions of the ultrastructural appearance of macrophages (Cline, 1975; Cheville, 1976) and were large pale cells with vesicular nuclei, a well developed Golgi zone, large mitochondria and numerous pinocytotic vacuoles containing heterogeneous material. The phagolysosomes were often packed with organisms resembling those seen in the epithelial cells, and showing evidence of degenerative change. The plasma membrane and cell wall of the organisms were usually clearly visible and occasionally discontinuous, while the cytoplasm was shrunken with electron translucent areas.

Bacteria within the epithelial cells were seen in very close proximity to the glands' lumina (Figure 100). Very occasionally bacteria were observed apparently being released from intact cells, although this was not a common feature. The increased cell loss noted histologically was confirmed ultrastructurally. Dying cells were seen in two forms. Some of the cells were shrunken in appearance, darker staining with pyknotic nuclei and present in the cytoplasm of these cells were bacteria resembling mucosalis (Figure 101).

These cells resembled apoptotic cells (Hopwood and Levison, 1976). They were seen at different stages in the process of loss from the epithelium. Initially they were situated between normal epithelial cells (Figure 101), to which they were joined by desmosomes, and at that time they did not protrude into the gland lumen. Later the cell was seen being squeezed out from between the two adjacent cells, so that part of the apoptotic cell protruded into the lumen and it lay between the upper region of the two adjacent cells to which it was still joined (Figure 104). At the next stage it lay along the surface of the epithelium, and not between adjacent cells, and the attachment to the previously adjacent cells by desmosomes could still be seen. Other degenerating cells had a swollen rather than a shrunken appearance (Figures 105 and 106), the cytoplasm was lighter staining and contained only a few bacteria. In these cells some of the bacteria were free and others were present in membrane-bound vesicles. The darker, shrunken cells seemed to contain more bacteria than the bloated, lighter cells.

The increased cell debris in the glands' lumina seen at a histological level could be better differentiated into cell types at an ultrastructural level. There were desquamated epithelial cells containing bacteria, neutrophil polymorphs and macrophages were also present and in these last two cell types bacteria were present in phagosomes or phagolysosomes (Figures 107 and 108). The neutrophil polymorphs had lobulated nuclei, poorly developed Golgi regions and endoplasmic reticulum, the cytoplasm contained a variable number of granules and few mitochondria were seen. In these features

they conformed to published descriptions (Cline, 1975). Bacteria morphologically similar to mucosalis were also seen free within the glands' lumina (Figure 100), presumably as the result of the breakup of degenerate epithelial cells, although bacteria were possibly also released from intact but damaged epithelial cells. Some of the free bacteria in the lumina appeared ultrastructurally normal, while others were shrunken and more electron dense. Collections of bacteria in free membrane-bound vesicles were also seen in the lumina of glands and could have resulted from the lysis of epithelial or phagocytic cells. The bacteria resembling mucosalis, which were present in phagosomes or phagolysosomes in phagocytic cells, showed evidence of degenerative change. Early in this process the resemblance of the bacteria to mucosalis, in the epithelial cells, was great but with digestion of the bacteria and condensation of the contents of the vacuole the bacteria could only be distinguished with increased difficulty. Both mononuclear and polymorphonuclear phagocytes were seen in the process of phagocytosis, engulfing bacteria in the lumina of glands (Figure 107). On one occasion in the gland lumen, a macrophage was seen apparently in the process of engulfing an epithelial cell (Figure 109). The epithelial cell was surrounded by the cytoplasmic protrusions of the phagocytic cell.

Macrophages containing bacteria were also seen within the epithelium, and in the surrounding lamina propria (Figure 99). The increase in cells in the lamina propria described histologically was confirmed ultrastructurally and the cells involved included lymphocytes, plasma cells, macrophages and a variable number of

eosinophil or neutrophil polymorphs (Figure 110). The ultrastructural features of the macrophages and neutrophils allowing recognition have already been described, and the eosinophils, plasma cells and lymphocytes conformed to published descriptions (Cline, 1975).

The late stage of the recovery phase

At the later stages of the recovery phase, when only a few isolated adenomatous glands were present, it was only these adenomatous glands which contained bacteria. Not all the cells in these glands contained bacteria and both goblet cells and more mature columnar epithelial cells, which lacked bacteria, were also present (Figure 111). The maturity of the cells containing bacteria varied and some of the cells with only a few bacteria had well developed microvillous brush borders, and other described features of maturity (Figure 112).

Within the lumina of the adenomatous glands at this time there were cells with bacteria; macrophages, neutrophils and desquamated epithelial cells. At this stage there were more macrophages and less neutrophils and the number of desquamated epithelial cells was reduced.

In the lamina propria surrounding these adenomatous glands there were macrophages containing bacteria resembling mucosalis, at various stages of degeneration within phagolysosomes. These macrophages were seen close to the basement membrane of the adenomatous glands, and also to those of the surrounding normal glands. The number of cells in the lamina propria was greater than at the earlier stages of the recovery phase and the cellular infiltration involved

all areas of the lamina propria. There were large numbers of plasma cells, lymphocytes and macrophages, and less neutrophil and eosinophil polymorphs, although occasionally eosinophils were present in substantial numbers.

It is stressed again at this juncture that this is a dynamic process and overlapping of the features from stage to stage are usually seen. In summary, early there are large numbers of mucosalis present within the epithelium and the cells are immature, later the bacteria begin to show evidence of degenerative change and are also present within cells of the phagocytic series. Associated with these changes the epithelial cells show increasing evidence of maturity and the mucosal architecture evidence of a return to normality.

Immunofluorescence Results

Controls

Two types of controls were used in this study. Tissue from normal pigs, where there was no gross or histological evidence of PIA and mucosalis was not recovered, and from typical cases of PIA (Chapter III, Part A).

In the normal pigs' intestine stained with either 1248/72 2C2 or 982/76 mucosalis anti-serum there was no specific fluorescence in the epithelium or other areas of the mucosa. When stained with R.A.P. there was some fluorescence in the epithelium, in which site it was confined to the crypt areas and was not seen on the villi. It was diffuse in nature rather than particulate, and although the staining was more intense in the apical cytoplasm it was not confined

exclusively to that region. In the lamina propria there were fluorescing cells, probably plasma cells.

The staining of tissues from typical PIA cases with mucosalis anti-serum has already been described in Chapter III. With tissues from which mucosalis serologically similar to strain 1248/72 2C2 has been recovered there is specific particulate fluorescence in the apical cytoplasm of affected intestinal epithelial cells when stained with 1248/72 2C2 mucosalis anti-serum, but not when stained with 982/76 mucosalis anti-serum. The reverse is also true, so that tissues from which mucosalis serologically similar to strain 982/76 has been isolated fluoresce when stained with 982/76 mucosalis anti-serum but not with 1248/72 2C2 mucosalis anti-serum. Only a small number of typical PIA cases have been stained with R.A.P. and in these most glands did not stain, but a few did show fluorescence. The fluorescence was diffuse not particulate, and concentrated mainly in the apical cytoplasm but was present throughout the cell. The staining was not as bright as in the tissue from the normal pigs. Few fluorescing cells were seen in the lamina propria, which was only sparse with the mucosa in fact made up predominantly of large adenomatous glands closely packed together.

For convenience the recovery phase of PIA will again be divided into three stages, but it is stressed once more that in this dynamic process division cannot always be made clearly and overlapping of the various features occurs.

The early stage of the recovery phase

When stained with 1248/72 2C2 mucosalis anti-serum there was often less fluorescence in the apical cytoplasm of the affected intestinal epithelial cells than had been expected from the numbers of organisms seen in silver-stained sections, and from the fluorescence seen in positive control tissues. Sometimes the fluorescence was more diffuse rather than particulate, as is usually seen in PIA. Although the fluorescence in the epithelium was mostly in the apical cytoplasm of the cells a little was seen away from this site, and deeper in the cells. Cells with specific intracellular fluorescence were also present in the lamina propria. The fluorescence was arranged around the periphery of these cells, which appeared to have a more centrally positioned nucleus, and these cells were probably macrophages.

If parallel cryostat sections were stained with 982/76 mucosalis anti-serum there was often a small amount of particulate fluorescence in the apical cytoplasm of some epithelial cells in occasional adenomatous glands. The fluorescence using 982/76 mucosalis anti-serum was always much less than in parallel sections stained with 1248/72 2C2 mucosalis anti-serum, in those animals from which mucosalis serologically similar to strain 1248/72 2C2 was recovered. There were also cells with intracellular fluorescence in the lamina propria, distributed in a similar manner to those in sections stained with 1248/72 2C2 anti-serum.

Sections stained with R.A.P. showed some particulate fluorescence in the apical cytoplasm of some cells in occasional adenomatous glands. This was superimposed on a more diffuse, less bright

fluorescence which involved the cells of some glands. The amount of particulate fluorescence, when stained with R.A.P., was usually small.

The intermediate stage of the recovery phase

Later in the recovery phase the amount of specific fluorescence in the epithelium, when stained with 1248/72 2C2 mucosalis anti-serum, was often further reduced. In those glands at the base of the mucosa with goblet cells present any fluorescence was confined to the non-mucus secreting epithelial cells (Figure 113). When stained with 982/76 mucosalis anti-serum there was a small, but variable amount of specific fluorescence in the apical cytoplasm of some affected epithelial cells.

When stained with 1248/72 2C2 mucosalis anti-serum the lumina of some glands showed specific particulate fluorescence (Figure 114), whilst some of this was intracellular some appeared to be free in the lumen. It was not possible to differentiate further the cells by this technique, but they probably correspond to the desquamated epithelial cells, macrophages and polymorphs seen by electron microscopy. Cells with specific intracellular fluorescence were also seen on the surface of the gland epithelium and these may have been epithelial cells in the process of being shed into the lumen, or phagocytic cells in close proximity to the gland epithelium. Some of these cells in the lumina of glands also showed fluorescence when stained with 982/76 mucosalis anti-serum.

The number of cells in the lamina propria with specific intracellular fluorescence, when stained with either 1248/72 2C2 or

or 982/76 mucosalis anti-serum, was increased at this stage. The fluorescence was mostly arranged around the periphery of these cells, which were probably macrophages. These cells were mainly positioned close to the basement membranes of the glands (Figure 114). Cells of this type were also seen within the epithelium of some adenomatous glands (Figure 115).

When parallel sections were stained with R.A.P. there was some particulate fluorescence in the apical cytoplasm of the epithelial cells in some of the adenomatous glands. The number of glands showing fluorescence was variable, but occasionally involved all of the glands. Cells in the glands' lumina, and in the surrounding lamina propria which had fluoresced when stained with mucosalis anti-serum appeared to fluoresce when stained with R.A.P. This fluorescence was particulate in nature. In addition in the lamina propria there were more cells with diffuse, rather than particulate, intracellular fluorescence which were probably plasma cells. The number of cells in the lamina propria fluorescing, when stained with R.A.P., was greater than earlier in the recovery phase.

The late stage of the recovery phase

In those cases with only isolated adenomatous glands present in the mucosa it was only these adenomatous glands which showed specific fluorescence in the epithelium when stained with 1248/72 2C2 mucosalis anti-serum. Often not all the cells in these glands fluoresced when stained with 1248/72 2C2 mucosalis anti-serum, some of the epithelial cells which failed to show fluorescence were goblet cells. The epithelium of the surrounding histologically normal

glands did not fluoresce. Within the lumina of these adenomatous glands there were cells with intracellular fluorescence as in the earlier stages, and occasionally also in the histologically normal glands. In the lamina propria there were large numbers of cells with specific fluorescence, arranged around the glands, both adenomatous and histologically normal glands.

Where the histological appearance was that of adenomatous glands "growing out", and the underlying mucosa had a normal histological appearance, on staining with 1248/72 2C2 mucosalis anti-serum, only the adenomatous epithelial cells contained intracellular fluorescence. In these glands the amount of fluorescence was small, and involved only some of the epithelial cells. Fluorescing cells were often present in the lumina of these adenomatous glands, and occasionally in the underlying histologically normal glands. In the lamina propria surrounding these adenomatous glands there were cells containing specific intracellular fluorescence, and they were also present in the lamina propria of the underlying histologically normal mucosa.

If parallel sections were stained with 982/76 mucosalis anti-serum cells in the glands' lumina and lamina propria showed specific intracellular fluorescence. Occasionally there was also a little particulate fluorescence in the epithelial cells of the adenomatous glands. There was no fluorescence in the epithelium of the histologically normal glands.

On staining parallel sections with R.A.P. there was some particulate fluorescence in the apical cytoplasm of some of the cells

in the isolated adenomatous glands and in the isolated adenomatous glands which were "growing out". These appeared to be the same glands that had shown fluorescence when stained with mucosalis anti-serum. In the surrounding and underlying histologically normal mucosa the epithelial cells of the crypts in the small intestine and the basal regions of the glands in the large intestine showed some diffuse fluorescence, involving all the epithelial cell but being brighter in the apical cytoplasm. In the lamina propria there were fluorescing cells. Cells with more diffuse fluorescence in the cytoplasm were considered to be plasma cells, and those with particulate fluorescence, macrophages. The cells in the lumina of the glands also contained particulate intracellular fluorescence. The cells in the glands' lumina, and those thought to be macrophages in the lamina propria corresponded to the cells which also showed fluorescence when stained with mucosalis anti-serum.

Bacteriological Results

In modified Z.-N. stained impression smears of mucosa taken in recovery phase cases with gross lesions of PIA clumps of acid-fast bacteria of vibrio morphology were seen both within epithelial cells and free. Some of the acid-fast bacteria appeared to be in vacuoles in the cells. Acid-fast bacteria were also seen within polymorphonuclear leucocytes.

From those cases showing gross lesions of PIA and corresponding to the early stage of the recovery phase mucosalis was recovered in similar numbers to those recovered from typical PIA cases. In the

intermediate stage only reduced numbers of mucosalis were recovered, or it was not recovered at all. This applied to tissue removed at necropsy from animals which had been examined immediately after euthanasia. In typical PIA large numbers of mucosalis were recovered from adenomatous tissues under these circumstances (Chapter III, Part A). Mucosalis was not recovered from pigs in the late stage of recovery.

If one compares pigs from herds A and D, with features of the recovery stage of PIA, then often mucosalis was recovered from pigs in herd A but not from pigs in herd D. These animals appeared to be at a similar stage in the recovery phase. The failure to isolate mucosalis from pigs in herd D with lesions of the recovery phase of PIA has been dealt with in Chapter III, Part C. A comparison of the recovery of mucosalis bacteriologically, and its demonstration by other techniques (modified Z.-N. stained impression smears, silver-staining, immunofluorescence and electron microscopy) from experimental cases (Chapter VII), and naturally occurring cases from herds A and D is presented in Table 9a.

The findings of this investigation of recovering PIA cases are summarised in Table 9b, where again the process is divided into early, intermediate and late stages.

Table 9a The Recovery and Demonstration of Mucosalis

Pig Reference	Phase of the Recovery Stage	<u>Mucosalis</u> Demonstrated	<u>Mucosalis</u> Recovered	Numbers of Viable <u>Mucosalis</u> per Gram of Mucosa
<u>Experimental Cases</u>				
183/76	T.S.I. and L.I. Intermediate	+	-	-
189/76	T.S.I. and L.I. Intermediate	+	+	T.S.I. 1.12×10^5 L.I. 6.8×10^4
206/76	T.S.I. and L.I. Late	+	-	-
207/76	T.S.I. and L.I. Late	+	-	-
208/76	T.S.I. and L.I. Late	+	-	-
<u>Herd A</u>				
1152/76	T.S.I. Early	+	+	1.1×10^6
20/75	T.S.I. Early	+	+	1.3×10^8
<u>Herd D</u>				
110/76	T.S.I. Early	+	-	-
240/76	T.S.I. Early	+	-	-
379/76	T.S.I. Early	+	-	-
405/76	T.S.I. Early	+	-	-
483/76	T.S.I. Early	+	-	-
20/77	T.S.I. Early	+	-	-

Table 9b Summary of the Features of the Recovery Phase of PIA

	Early	Intermediate	Late
Bacteriology	<u>Mucosalis</u> recovered in numbers equivalent to those in typical PIA.	<u>Mucosalis</u> not recovered, or recovered in numbers lower than from typical PIA.	<u>Mucosalis</u> not recovered.
Gross Findings	Lesions typical of PIA.	Lesions typical of PIA.	Slight thickening, or no gross lesions.
Histological Findings.	Affected areas of mucosa are adenomatous. At the base of some glands occasional goblet cells are seen. Increased cell debris in lumina of glands.	Mucosa adenomatous. Goblet cells present in more basal glands. Increase in luminal cell debris. Increase in cells in lamina propria.	Only occasional isolated adenomatous glands seen. In the small intestine there is villous atrophy. Glands "growing out" and underlying mucosa normal in appearance and devoid of bacteria.
Ultrastructural Features	Bacteria mostly normal in appearance. Occasional bacteria show evidence of degeneration, and phagolysosomes containing bacteria sometimes seen. Less often bacteria present in macrophages in the lamina propria.	Many bacteria show degenerative changes and are aggregated in clumps. Bacteria present in membrane-bound vesicles. Bacteria seen in phagocytic cells in the lumen, the epithelium and the surrounding lamina propria.	Only affected glands contain bacteria, and only some cells in these glands. Many cells more mature. Bacteria also present in phagocytic cells in the lumina of glands, and in the surrounding lamina propria.
Immunofluorescence	Fluorescence in the adenomatous glands variable. Cells with specific intracellular fluorescence in the lamina propria probably macrophages.	Variable fluorescence in the adenomatous epithelium. Specific fluorescence in cells in the lumina of glands and the surrounding lamina propria.	Only affected glands show specific fluorescence, and only some cells in these glands. Specific fluorescence present in cells in the glands' lumina and the surrounding lamina propria.

DISCUSSION

This is the first reported study of the recovery phase of PIA. Although further work will be necessary to clarify many of the observations, the report gives some indication of the mechanisms involved in recovery from the condition and the resolution of the lesions.

Coincident with a clinical improvement in affected animals there was a failure to recover mucosalis from the lesions, although its presence could often be demonstrated by a number of techniques. Ultrastructural changes in the bacteria were seen at this stage, and could be morphological indication of damage precluding growth of the organism in vitro. The bacteria in epithelial cells were also seen within membrane-bound vesicles, phagosomes or phagolysosomes, in varying states of degeneration. Antibodies may play some part in these bacterial changes. Associated with these changes in the bacteria the epithelial cells showed evidence of increasing maturation, there was an increased rate of epithelial cell loss from the mucosa, and return of the mucosal architecture to normal. There was also an influx of phagocytic cells, macrophages and neutrophil polymorphs, and in the lamina propria there was an increase in the number of lymphocytes and plasma cells. The maturation of the epithelial cells on the disappearance or killing of mucosalis and the resolution of the lesions is further evidence that the presence of mucosalis is intimately associated with the immaturity of the epithelial cells, and the development of adenomatosis. It also adds support to the hypothesis that it may be of aetiological significance.

On occasions when mucosalis was not recovered the bacteria did not appear to differ morphologically from those seen in typical PIA cases. The absence of ultrastructural evidence of degeneration in some cases does not preclude that the bacteria were damaged. Van Houte et al. (1977) reported the killing of E. coli by a granulocyte fraction without recognisable ultrastructural alterations. The mechanisms responsible for the loss of viability of mucosalis were not clearly identified, but the presence of antibody possibly closely linked to the bacteria was indicated by immunofluorescence studies. The release of lysosomal constituents by neutrophil polymorphs, which were present, possibly also played a part but whether these would be active on organisms within enterocytes is unknown.

The relationship between the intestinal epithelial cell and mucosalis in PIA is very different from previously described host-parasite interactions and therefore it is not possible to exactly equate these findings with reported observations on other conditions and infective agents. An attempt will be made, however, to compare these observations on the recovery phase of PIA with widely held views on related subjects.

The immunity to intracellular parasites has been extensively investigated, and also the immunity to bacteria, including those infecting mucous membranes. In general it is considered that humoral antibodies are particularly effective in combating systemic infections by bacteria that replicate outside cells and thus are free in body tissues and fluids, while cell-mediated immunity is reported to be the major mechanism for destroying bacteria that replicate

intracellularly and are thus protected from serum antibodies (Campbell, 1976). Published work on immunity to intracellular parasites has concentrated on organisms within cells of the mononuclear phagocytic series, the so-called professional phagocytes. In the enterocyte we are dealing with a cell which has a degree of phagocytic capability, but would probably be considered a non-professional phagocyte (Jones, 1975). In cell-mediated immunity interactions between mononuclear phagocytes and sensitized T-lymphocytes occur on exposure to antigen, with the result that the former are attracted to the site of the antigen (McGregor and Logie, 1975). In addition, lymphocytes specifically committed to microbial antigens, can be stimulated to release products which enhance the endocytic and microbicidal capacity of macrophages. T-cells may also operate directly, expressing cytotoxicity to the antigen (Campbell, 1976). Sensitized lymphocytes contain a factor which inhibits the growth of Mycobacterium tuberculosis within macrophages in vitro (Turcotte, Des Ormeaux and Bonduas, 1976). Since mucosalis resides intracellularly it is tempting to speculate that cell mediated immunity may be important in immunity to infection with the organism. Mucosalis is free within the cytoplasm, while most of the other intracellular bacteria described are located within phagolysosomes, in which case they appear to be resistant to lysosomal enzymes, or within phagosomes, when their presence apparently prevents fusion of the phagosome with lysosomes. With Toxoplasma the addition of anti-toxoplasma antibody before uptake by macrophages resulted in the destruction of the organism within these cells (Janes, Len and Hirsch, 1975), but if the antibody was added after the organisms had entered the macrophages then there was no effect on multiplication

and survival of the *Toxoplasma* within these cells. This is probably due to the fact that antibodies, if they enter the cell, are unable to reach the phagosome containing *Toxoplasma*. Antibodies, however, pass through the intestinal epithelial cell normally and this, along with the fact that *mucosalis* is free within the cytoplasm, may be of significance, allowing specific antibodies access to *mucosalis*.

Again, working with *Toxoplasma*, Borges and Johnson (1975) showed that supernatants from T-lymphocytes, which inhibited *Toxoplasma* multiplication in monocytes, did not influence parasite growth in HeLa cells. For *Shigella flexneri*, in vitro, MacDermott et al. (1976) have demonstrated antibody-dependent, cell-mediated cytotoxicity in a complement free system. In the presence of specific antibody, lymphocytes, as well as macrophages and granulocytes, could effectively kill the organism. Lymphocytes and macrophages were ineffective in the absence of specific antibody, in contrast to granulocytes which killed some shigella in the absence of specific antibody, although a higher percentage killing occurred if specific antibody was present. *Shigella* is an intracellular parasite of the enterocyte, in some ways comparable to *mucosalis*.

Local secretory immunity operating at mucous membranes is now well recognised (Tomasi, 1976; Brandtzaeg and Baklien, 1976), and the immunoglobulins present in mucous surfaces differ from serum antibodies. Secretory IgA usually predominates, although in the younger animal - certainly in the pig (Allen and Porter, 1973 and 1977), IgM contributes significantly and in the unweaned animal may be present in higher concentrations than IgA. It has been demonstrated

that both IgM and IgA are located in and transported through the intestinal epithelial cells, where linkage to secretory component takes place (Brandtzaeg and Baklien, 1976). Local cell-mediated immunity has been described (Waldman and Ganguly, 1975), and is analogous to systemic cell-mediated immunity.

The role that secretory immunoglobulins play in the recovery phase of PIA is unknown, although that they were present on the bacteria was indicated by immunofluorescence studies. The antiserum used in this study was not directed specifically against particular immunoglobulin classes, but was rather against all classes. Its presence intracellularly in epithelial cells would, however, suggest that it was probably IgA and/or IgM. As well as the demonstration of immunoglobulins in association with mucosalis the presence of increased numbers of plasma cells in the lamina propria provide further evidence that immunoglobulins are involved in the recovery from PIA. The presence of antibody to mucosalis in the serum of affected pigs has also been reported (Rowland and Lawson, 1974). As early as thirteen days after oral dosing with mucosalis, mucosalis antigen was detected in macrophages in the lamina propria (Chapter IV), and bacteria may on occasions be recovered from the mesenteric lymph node (Rowland and Lawson, 1974), and this could initiate systemic antibody production. On the basis of the evidence obtained so far it would appear that antibody to mucosalis is produced and enters the epithelial cell, inhibition of mucosalis occurs, the epithelial cells mature and are lost from the mucosa and along with them mucosalis. The apparent clumping of mucosalis seen ultrastructurally may be a manifestation of the presence of antibody on the bacteria.

With respect to the time course of immunoglobulin production in enteric bacterial disease most studies have dealt with surface or luminal organisms and not with intracellular bacteria. For example, with E. coli in young pigs (Porter et al. 1974) immunoglobulin production occurred within a few days and peaked at seven to ten days. The experimental system of Porter et al. used a single, large dose of E. coli, and thus differed from PIA in many ways. Recovery from PIA and mucosalis infection in the experimental disease (Chapter VII) was approximately eight to nine weeks after oral infection. It is probable that this length of time is necessary for the growth and release of sufficient mucosalis antigen to stimulate an adequate immune response. Pierce and Gowans (1975) found using cholera toxin that oral priming was relatively inefficient in evoking antitoxin producing cells in the mucosa and required the prolonged administration of large amounts of antigen. Recent studies on the chronology of development of antispirochaete antibody in swine dysentery are of interest. Using an indirect fluorescent antibody technique, serum antispirochaete antibodies were detected initially at four weeks after the onset of diarrhoea, in swine exposed to infective swine dysentery inoculum (Lee and Olson, 1977). In this experimental situation the incubation period is on average eleven days (five to twenty four days) (Olson, 1974). The timing of the production of antispirochaete antibody in experimentally produced swine dysentery does not differ greatly from the time course of recovery in experimental PIA, which is apparently, at least in part, antibody mediated.

The immune-mediated emigration of neutrophils into the intestine has been described and may be part of the intestinal immune

response, especially in relation to bacteria (Bellamy and Nielsen, 1974a and 1974b, Bellamy and Hamilton, 1977). Neutrophils were present in the lumina of glands during the recovery phase of PIA and mucosalis was seen in varying stages of degeneration within phagolysosomes in these cells. Neutrophils are attracted to antigen-antibody complexes (Henson, 1971a and 1971b), which is interesting in light of the demonstration of immunoglobulins on mucosalis in these studies. The ultrastructural appearance of mucosalis within the phagocytic cells, both macrophages and neutrophil polymorphs, is consistent with them being effectively destroyed by these cells, and is in accordance with the crucial role played in host defence by these cells in removing micro-organisms and other deleterious substances (Van Furth, 1974). Neutrophil lysosomal enzymes can be released by cell death or exocytosis (Cheville, 1976). Interaction with antigen-antibody complexes results in the release of lysosomal enzymes, without rupture of the cell (Henson, 1971a and 1971b). These enzymes may have played a part in the variable damage seen ultrastructurally to some epithelial cells, and possibly also to mucosalis. Macrophages are reported to have a more complex role (Van Furth, 1974) including the ingestion of cell debris, micro-organisms and antigen-antibody complexes and this is consistent with the findings described here.

The immunofluorescence studies reported here do not differ from published accounts of the demonstration of immunoglobulins within the cytoplasm of intestinal epithelial cells in the pig (Allen and Porter, 1970, 1973; Allen, Smith and Porter, 1976; Brown and Bourne, 1976a, 1976b). Immunoglobulins are present in the apical

cytoplasm of crypt cells but not mature epithelial cells. Although the anti-serum used did not distinguish between the different immunoglobulin classes the findings of the present author agree with the distribution of immunoglobulins in the intestinal epithelium of normal pigs. Further work will be necessary using specific antisera to try to elucidate the class(es) of immunoglobulin involved in immunity to mucosalis in the intestinal epithelium.

Immunity to neoplastic cells has been extensively investigated in recent years and both humoral and cell-mediated responses have been described. Cell-mediated immune reactions with destruction of tumour cells are considered to be the most important response against tumours (Calman, 1975). Immune T-lymphocytes can be directly cytotoxic and antibody dependent cellular cytotoxicity involving mononuclear phagocytes, K-cells (Lymphocytes) or polymorphonuclear leucocytes has been described (Oleske et al., 1977). Neoplastic cells often express unusual antigens, to which a humoral immune response may be mounted, and in the case of viral induced tumours there may be expression of viral antigen(s) on the cell surface. It is not at present known if there is any alteration in the antigenicity of the adenomatous epithelial cells in PIA, or the expression of mucosalis antigen(s) at the cell surface. In the sera of some pigs affected with PIA there is antibody to intestinal epithelial cells (Lawson, personal communication, 1977). In recovering cases dying cells were seen in the process of being shed from the epithelium, and disintegrating cells were present in the glands' lumina. It is possible that these cells had been damaged in the immune response. Although cells are continually lost from the intestinal

epithelium under normal circumstances as they age, it is unusual to observe them in the number seen in this study.

The presence of secretory immunoglobulins in human colonic neoplasms has been investigated (Weisz-Carrington, Poger and Lamm, 1976). These workers found that when compared with normal tissue, neoplastic epithelium contained less immunoglobulin, the decrement being usually inversely parallel to the degree of differentiation. So benign polyps closely resembled normal colonic mucosa in secretory immunoglobulin content, in contrast to atypical areas of benign polyps and carcinomas which exhibited greatly reduced or no such secretion. In many of the carcinomas there was a reduced number of plasma cells in the stroma, which the authors suggest is due to an alteration in the normal mechanism for attracting the circulating precursors of local IgA plasma cells. In villous polyps, especially where the epithelium was pseudostratified and composed of cells of poor polarity, the glands exhibited atypical patterns of immunofluorescence. Such areas were often negative for secretory component (S.C.) and intra-epithelial immunoglobulins and showed normal or decreased numbers of plasma cells. The synthesis of S.C. by colonic neoplasms has also been studied (Poger, Hirsch and Lamm, 1976). S.C. is present normally in the apical cytoplasm of non-mucus secreting crypt cells. In general it was found that tumours producing S.C. were more differentiated, but the correlation between S.C. and the presence of goblet-like mucin-producing cells was better still, even though the latter themselves do not contain S.C. These findings are of interest when compared to those cases of typical PIA examined in this study. In most of the adenomatous glands the

epithelium did not contain intracellular immunoglobulins and there were only a few immunoglobulin producing cells in the lamina propria. It is not known, if as suggested by Weisz-Carrington et al. that this represents a failure of the epithelium to attract secretory immunoglobulin producing plasma cells. Neither is it known if there was defective transport of immunoglobulins from the plasma cells into the epithelium. The large number of epithelial cells and the presence of only a small number of plasma cells may have meant that the concentrations of immunoglobulins in each cell were too low to be detected with the reagents and techniques used in this study. Many of the glands in PIA resemble the villous polyps described by Weisz-Carrington et al., in having a pseudo-stratified epithelium. In both cases the presence of intracellular immunoglobulins was not demonstrated. This could be due to the fact that the concentration of immunoglobulin in each cell is too low to be detected in contrast to the normal situation with a large amount of immunoglobulin in each of a small number of cells, or to a defective transfer to the epithelial cells, possibly due to the lack of polarity. The observation by Poger et al. that the production of S.C. is correlated to the presence of goblet-like mucin-producing cells could be important. The presence of S.C. in PIA has not been investigated, but if the situation is similar to that in human colonic neoplasms, this may explain the absence of immunoglobulin from the epithelial cells, since S.C. is necessary for uptake of the IgA and IgM by these cells (Brandtzaeg and Baklien, 1976). This would, however, pose the problem as to how the immunoglobulins reach mucosalis

in the epithelial cell during the recovery phase of PIA, when immunoglobulins were demonstrable in the epithelium and large numbers of plasma cells were present in the lamina propria.

The cellular infiltrate in neoplasms has been described (Underwood, 1974). For breast tumours the greater the macrophage and plasma cell infiltration the better the prognosis, and the lower the risk of developing metastases (Lauder *et al.*, 1977). Macrophages have been studied in most detail; in ultrastructural studies they have been seen ingesting apparently intact tumour cells (Carr *et al.*, 1974; Broome, Potter and Carr, 1976), and are cytotoxic to tumour cells (Evans and Alexander, 1972). A soluble product of lysosomal origin has been suggested as the active agent (Currie and Basham, 1975) in this cytotoxicity. In one instance in a recovering PIA case a macrophage was seen possibly engulfing an intestinal epithelial cell, and the role of the macrophage in the recovery phase of PIA would seem to warrant further study.

The ultrastructure of mucosalis within intestinal epithelial cells during the recovery phase of PIA is interesting in that it was seen either singly or in groups in membrane-bound vesicles. In this feature it differs from typical PIA, but more closely resembles the relationship between the intestinal epithelium and other enteric micro-organisms (Staley, Janes and Corley, 1969b; Takeuchi, 1971 and 1975). This further emphasises the uniqueness of the more usual relationship between mucosalis and the epithelial cell. At some time during the recovery phase mucosalis appears to lose the ability to maintain this special position.

Once elimination of mucosalis has taken place, resolution of the lesions involves considerable remodelling of the epithelium before the mucosa returns to its normal appearance. This process may be facilitated by the normal rapid intestinal epithelial cell turnover. As part of the cyclic changes occurring in the human endometrium, at some stage atrophy of the epithelium occurs, and recently this atrophy has been related to apoptosis (Hopwood and Levison, 1976). Apoptosis is the process of "controlled cell deletion" (Kerr, Wyllie and Currie, 1972; Wyllie *et al.*, 1973; Wyllie, Kerr and Currie, 1973) applied to individual cell death. Hopwood and Levison described apoptotic bodies basally situated within epithelial cells, within macrophages in the epithelium and stroma, and less commonly in the extracellular space or in the glandular lumina. They suggest that apoptotic cells and debris are ingested by macrophages which pass the basement membrane, and these cells then cross back into the stroma, or alternatively apoptotic cells or cell debris passes into the stroma and is ingested. In recovering PIA cases apoptotic cells were seen in the epithelium and at various stages in transit from the epithelium into the gland lumina. Apoptotic bodies were also seen in the epithelium, mostly within epithelial cells where they were predominantly basally situated, and also apparently intercellularly. These bodies varied considerably in size and morphology, and were also seen free in the gland lumina, in phagocytic cells in the gland lumina and within macrophages in the lamina propria. They were also seen apparently free within the lamina propria and in the process of transfer from the epithelium into the lamina propria. These findings are consistent with previously described instances of the occurrence

of apoptosis. Apoptosis has been described as occurring normally in the intestinal epithelium (Cheng, 1974; Searle et al., 1975; Potten and Allen, 1977), and it is increased after the administration of cancer-chemotherapeutic agents (Searle et al., 1975) and after X-irradiation (Hugon and Borgers, 1966; Ghidoni and Campbell, 1969) or during epithelial regeneration after ischaemic necrosis of the intestinal villi (Pitha, 1969). The extrusion of apoptotic bodies into the gland lumen (Hugon and Borgers, 1966) or into the lamina propria (Ghidoni and Campbell, 1969) has been described. Uptake of apoptotic bodies by adjacent epithelial cells is also documented (Hugon and Borgers, 1966; Ghidoni and Campbell, 1969; Cheng, 1974; Cheng and Leblond, 1974; Searle et al., 1975). Apoptosis is also recognised in tumours (Kerr, Wyllie and Currie, 1972; Kerr and Searle, 1972a) and it is suggested that it is important in therapeutically induced tumour regression (Kerr and Searle, 1972b; Kerr et al., 1972). Its occurrence has been described in the adrenal gland after the removal of adrenocorticotrophic hormone (Wyllie et al., 1973).

Some of the membrane-bound vesicles containing osmiophilic material resembled autophagolysosomes, which develop from the Golgi complex and engulf degenerate cellular organelles (Cheville, 1976). Autophagy is the process whereby damaged cellular organelles are removed from the cell (Cheville, 1976) and it is a common reaction in sublethally altered cells (Arstila and Trump, 1968; Arstila et al., 1971). Cell atrophy occurs by autophagocytosis (Cole et al., 1971). Although occurring in almost all normal cells its rate is increased under certain circumstances; remodelling after metamorphosis and development, during differentiation and ageing, and

following hormonally induced changes in tissues and cells (Arstila and Trump, 1968; De Duve and Wattiaux, 1966). Autophagic vacuoles are formed by the enclosure of a portion of the cytoplasm with organelle(s) by cisternae of smooth surfaced endoplasmic reticulum (Arstila and Trump, 1968). The double-walled sac formed fuses with primary or secondary lysosomes, and acid hydrolases are released into the space between the two membranes. The inner of these membranes first thickens and is then digested completely to form the single membrane limited autophagolysosome. In some of the autophagosomes in the epithelial cells in recovery PIA cases mucosalis was also present and perhaps these should strictly be termed ambiphagosomes (De Duve and Wattiaux, 1966), as they contain external bacterial material as well as cellular organelles. The fact that mucosalis is free within the epithelial cell cytoplasm under normal circumstances without invoking changes may mean that it is in fact treated in a similar manner to cellular organelles. The groups of mucosalis in membrane-bound vesicles in the epithelial cells may be the result of such a response, i.e. "autophagocytosis" of damaged mucosalis, and if there is also epithelial cell damage the autophagolysosomes may contain damaged true cellular organelles as well as mucosalis.

Immunity in other conditions associated with campylobacters has been investigated. In bovine venereal vibriosis caused by Campylobacter fetus subsp. fetus, there is a transient infertility in the female, associated with an endometritis. Bacteria are usually cleared from the oviducts and uterus forty to sixty days following infection (Corbeil et al., 1975a), but the cervicovaginal carrier state often persists for several additional months. Local

secretions contain C. fetus antibodies (Corbeil et al., 1974), mononuclear and polymorphonuclear phagocytes may also play a part in immunity (Corbeil, Corbeil and Winter, 1975) and cell-mediated immunity has been demonstrated (Corbeil, Corbeil and Winter, 1975). Wilkie and Winter (1971a and 1971b) and Wilkie, Duncan and Winter (1971) demonstrated C. fetus in epithelial and sub-epithelial cells of infected organs by immunofluorescence and electron microscopy. In the only published electron micrograph (Wilkie and Winter, 1971b) it is difficult to be certain that the bacteria-like structure is C. fetus. These authors also describe C. fetus within vacuoles in epithelial cells. The presence of bacterial antigen within the epithelium and sub-epithelial mesenchymal cells is similar to the recovery phase of PIA. An ultrastructural study of the endometrial inflammatory lesions in bovine venereal vibriosis has been reported (Corbeil et al., 1975a). Most of the identifiable intraluminal cells were degenerating polymorphs, some of which contained phagosomes with varying contents, and there were a few mononuclear phagocytic cells. Bacteria within these cells were not described in contrast to the recovery phase of PIA. There were focal cellular accumulations in the endometrium, mainly of mononuclear cells; T- and B- lymphocytes were the most abundant, plasma cells were the next most frequently seen cell type and mononuclear phagocytes were observed least often. These mononuclear phagocytes contained phagocytosed material, and although no reference is made to the fact, in the illustrations some of this material appears as if it could have been of bacterial origin. C. fetus is killed by polymorphs and macrophages in the presence of antibodies (Corbeil, Corbeil and Winter, 1975). It

can be seen that the lesions of bovine venereal vibriosis have features in common with the recovery phase of PIA.

The differences in the recovery of mucosalis from pigs in herds A and D, with similar histological and ultrastructural lesions, is unexplained. This could be due to differences in the mucosalis strains involved in the two herds. Differences in sensitivity to serum factors, measured as killing of the organism has been described for shigella (Reed and Albright, 1974; Reed, 1975), Neisseria gonorrhoea (Tramont, Sadoff and Wilson, 1977) and E. coli (Glynn and Howard, 1970). In E. coli the differences in serum sensitivity have been related to the character of the K-antigen of the organism.

In summary the recovery phase of PIA has been studied. The elimination of mucosalis and the resolution of the adenomatous lesions were described. Some of the factors involved in this, and possible mechanisms playing a part in the recovery have been discussed. Further work will be necessary to confirm and extend these findings, and to further investigate the immune mechanisms involved. This study indicates the directions which this work could take.

CHAPTER X

STUDIES ON PROLIFERATIVE HAEMORRHAGIC ENTEROPATHYINTRODUCTION

Proliferative haemorrhagic enteropathy (PHE) was described in association with PIA by Rowland and Rowntree (1972). The underlying adenomatous change present in both conditions was stressed by Rowland and Lawson (1975a) and Rowland, Lawson and Roberts (1976), and the demonstration of mucosalis by immunofluorescence and electron microscopy reported. PHE was described in Australia (Kelly and Cameron, 1976) and intracellular bacteria of vibrio morphology demonstrated but not isolated. More recently Love, Love and Edwards (1977) described the recovery of mucosalis from cases of PHE, also in Australia. Lawson, Rowland and Roberts (1977a) although able to recover mucosalis from cases of PHE commented that the numbers recovered were lower than would have been expected considering the number of organisms seen by other techniques and the freshness of the material. The numbers of mucosalis isolated by Love et al. from PHE cases in Australia were also small (Love, personal communication, 1977), and the strain of mucosalis recovered by these authors closely resembles serologically and biochemically the neotype strain (Lawson, personal communication, 1977). Mucosalis has also been recovered from adenomatosis cases with haemorrhage in America in similar numbers to that recovered from PIA cases (McAllister, personal communication, 1977).

The acuteness of the clinical disease in PHE prompted Rowland and Lawson (1975a) to suggest that it may be due to infection with mucosalis in a fully susceptible population. Its occurrence and behaviour in minimal disease herds (O'Neill, 1970; Rowland and Rowntree, 1972; Love, Love and Edwards, 1977) support this concept. O'Hara (1972) suggested that the histological lesions were older than the brief clinical illness suggested.

In this chapter a study of PHE cases is reported, which was carried out in an attempt to determine why mucosalis was not readily recovered from such cases and to try to shed light on the pathogenesis of the condition.

MATERIALS AND METHODS

Animals

The animals which provided material for this study were naturally occurring cases of PHE in animals at the Meat and Livestock Commission (M.L.C.) boar testing stations at Selby and Stirling. Details of the animals are presented in Table 10a. In the case of the animal from Stirling, examination was carried out immediately after death. With the animals from Leeds the samples were taken at death, frozen and sent in an insulated container containing dry ice. They were received eighteen to twenty four hours after death of the animal and were still frozen. Fixation and examination were carried out immediately after thawing at room temperature.

Control tissues were obtained from normal pigs, without gross or histological evidence of PIA and from which mucosalis was not

Table 10a Details of the PHE Cases Examined.

Pig Reference	Origin	Weight and Age	Sex	Died or Killed	Time Between Death and Examination	Recovery of <u>Mucosalis</u>
84/75	Leeds	214 lbs 167 days	Male	Died	48 hours Sent frozen, on dry ice	-
52/76	Leeds	83 lbs 94 days	Male	Died	24 hours + Sent frozen, on dry ice	-
96/76	Stirling	100 lbs 98 days	Female	Killed	Eviscerated 1½ hours after slaughter. Tissues processed 1¼ hours after sampling.	+ 2 2.4 x 10 ² organisms per gram of mucosa.
108/76	Leeds	209 lbs. 166 days	Male	Died	26½ hours Sent frozen, on dry ice	-

recovered, and from the typical cases of PIA described in Chapter III, Part A.

Gross Examination

Only the alimentary tract or portions of the intestines were available for examination.

Bacteriological Examination

The affected mucosa was examined as described in Chapter II.

Histological and Electron Microscopic Examination

Tissues taken from the areas adjacent to those examined bacteriologically were fixed and processed as described in Chapter II.

Immunofluorescence Examination

This was carried out as described in Chapter IX, using 1248/72 2C2 mucosalis anti-serum, 932/70 mucosalis anti-serum and F.I.T.C. conjugated rabbit anti-pig γ -globulin (R.A.P.).

RESULTS

Pathological Results

Gross Findings

Lesions were restricted to the terminal ileum, variable lengths (50 to 100 cms.) of which were involved. There was reticulation of the serosal surface and the affected portions of intestine were firmer to handle when compared with uninvolved areas. The mucosa showed exaggerated folds and the lumen contained free blood and fibrin.

Often a fibrin cast was present in the lumen. No focal areas of haemorrhage could be seen with the naked eye. The large intestine usually contained changed blood throughout its length. No thickening of the mucosa of the large intestine was seen in the cases examined.

Histopathology

Histologically the affected areas of small intestine had an adenomatous appearance (Figure 116). This contrasted with the normal villous architecture in the more proximal uninvolved portions. At the base of the mucosa in some glands there were occasional goblet cells (Figure 117), and less often histologically normal glands were seen in this site underlying the adenomatous tissue. The lumina of the glands contained a large amount of cellular debris, including many neutrophil polymorphs (Figure 116). Epithelial cells were being extruded from the mucosal surface and in these areas free red blood cells were visible in the intestinal lumen, in close proximity to the mucosa (Figure 118). The histological appearance of the large intestine was unremarkable.

Many of the epithelial cells stained less distinctly than in normal tissue or typical PIA cases, and densely eosinophilic, shrunken, necrotic epithelial cells were seen. Some of the necrotic cells had apparently been undergoing cell division at the onset of degeneration. There were elongate cells with dense spindle-shaped nuclei present, and also broader cells with open vesicular nuclei. Often the former cells were deeply eosinophilic and necrotic.

In some areas there was attempted villus formation (Figure 119),

resembling that seen in the recovery phase of PIA. Also adenomatous glands were seen adjacent to the luminal surface, with the underlying mucosa having a normal histological structure, so that the adenomatous glands appeared to be "growing out".

At the base of the epithelial cells in some of the adenomatous glands there were dense eosinophilic staining bodies. These were mostly positioned below the nucleus in the cell and resembled the apoptotic bodies, described by Hopwood and Levison (1976). They resembled the inclusions seen in recovering PIA cases and varied in size from approximately one twentieth to one quarter the size of the nucleus, and they were argyrophilic in silver-stained sections. In Feulgen stained sections they were Feulgen positive, indicating the presence of D.N.A.

In silver-stained sections there were large numbers of vibrios in the apical cytoplasm of epithelial cells in the adenomatous glands. Although predominantly in the apical cytoplasm silver-stained vibrios were also seen deeper in the cell. The organisms had the appearance of being clumped within the cells. Where epithelial cell loss from the surface was occurring, the cells being shed contained argyrophilic vibrios. The cells in the lumina of the glands also contained silver-staining, vibrio shaped bacteria. Although it was not possible to identify these cells histologically they probably included desquamated epithelial cells, macrophages and neutrophils as seen by electron microscopy. Free vibrios were also seen in the lumina of the glands. Cells containing intracellular silver-stained bacteria were also present in the lamina propria, adjacent to the adenomatous glands. In these cells, which were probably macrophages, the bacteria were often clumped together and were arranged peripherally in the

cytoplasm, around a central nucleus. Similar cells were also seen within the epithelium. In areas where attempted villus formation was occurring the more basally situated epithelial cells of the villi contained vibrios but those towards the villus tip contained few, or were free of vibrios. Where the mucosa had the appearance of adenomatous glands "growing out", vibrios were present within the epithelium of these adenomatous glands but not of the underlying histologically normal glands. Vibrios were, however, present within cells in the lamina propria surrounding these histologically normal glands and occasionally within cells in the gland lumen.

There was an increase in the number of cells in the lamina propria in comparison to typical PIA and normal intestine. Macrophages, plasma cells and lymphocytes were present in large numbers (Figure 120). A variable number of eosinophil and neutrophil polymorphonuclear leucocytes were also present. These last two cell types were seen in close proximity to the glands and when present in the lamina propria were usually also seen in the epithelium and lumina of the glands.

Electron Microscopy

In affected glands the epithelial cells contained very large numbers of bacteria, resembling mucosalis. These were situated in the apical cytoplasmic region of the cell, which was usually packed with them (Figure 121). The epithelial cells ultrastructurally resembled those described in PIA, except that there was evidence of damage to many of them. The cells had apical secretory granules, abundant ribosomes and a poorly developed membrane system. Many of

the mitochondria were swollen and ballooned, with disintegration of cristae and the formation of dense matrix granules. The cytoplasm was lighter staining than in both normal intestinal epithelium and adenomatous epithelium in typical PIA, and the cell and organelle outlines were often less distinct. The cells were swollen and in some areas the cell wall was incomplete with leakage of cytoplasm, intracellular organelles and bacteria into the lumen (Figure 121). Other cells had blebs of cytoplasm protruding from their luminal surfaces. In some glands there were occasional goblet cells, and these mucus-secreting cells did not contain bacteria (Figure 122).

Shrunken, necrotic cells were seen, in which the cytoplasm was more densely stained and packed with bacteria, some of which were in membrane-bound vesicles, and the nucleus was pyknotic. These cells were situated between adjacent viable cells in the gland and rested basally on the gland basement membrane. They were also seen in the process of extrusion from the epithelium and during this procedure they were observed at varying levels in the epithelium. The impression gained was that they were being squeezed out from between the adjacent cells until they came to lie on the surface of the gland epithelium, from which position they were shed into the lumen. These dark, shrunken cells resembled the apoptotic cells described by Hopwood and Levison (1976), which were also described in the recovery phase of PIA (Chapter IX).

Some of the epithelial cells contained complex phagolysosomes, probably autophagolysosomes (Cheville, 1976) in which remnants of cell organelles and occasional organisms could be identified. The Feulgen positive, eosinophilic bodies described histologically in the

epithelium were seen as dense osmiophilic bodies situated predominantly basally within the epithelial cells. They resembled apoptotic bodies (Kerr, Wyllie and Currie, 1972) and were also seen in other areas of the epithelial cells, but less frequently. Macrophages within the epithelium, and in the immediately adjacent lamina propria also contained these bodies, which were also seen apparently free in intercellular spaces of the epithelium. They were infrequently present in the lumina of the glands, both free and in macrophages. They appeared mostly to be within epithelial cells or macrophages in the epithelium, or macrophages in the surrounding lamina propria, but were occasionally lost into the gland lumen.

The lumina of many of the glands contained an abundance of cell debris released from damaged epithelial cells, and cells, including desquamated epithelial cells containing bacteria, macrophages and large numbers of neutrophils. Both the neutrophils and macrophages contained bacteria resembling mucosalis, in membrane-bound vesicles, phagosomes or phagolysosomes (Figures 123 and 124). The bacteria were in varying stages of disintegration within these vesicles. The numbers of mucosalis in each phagosome varied from single organisms up to as many as twelve. These bacteria varied and in some the outer wavy double membrane was more clearly visible due to separation from the underlying plasma membrane. In many the cytoplasm showed electron translucent areas, with the remnants of cytoplasm arranged in clumps along the inner surface of the plasma membrane. In some organisms the cytoplasm had a more granular appearance, while other bacteria were more shrunken and electron dense. As digestion proceeded it became increasingly difficult to recognise mucosalis, and only dense shrunken bodies, remnants of bacterial

cytoplasm and fragments of outer membrane were seen. Some of the macrophages in the lumina also contained apoptotic bodies. Free vibrios were seen in the glands' lumina, and these resembled those seen in the epithelial cells. Both mononuclear and polymorphonuclear phagocytes were seen in the process of ingesting bacteria resembling mucosalis. Collections of mucosalis within free, membrane-bound vesicles were seen in the lumina of glands and presumably resulted from the disruption of epithelial or phagocytic cells.

The bacteria in the apical cytoplasm of the epithelial cells, which resembled mucosalis, had the characteristic outer wavy double membrane. Many of them were swollen with clear electron translucent areas in their cytoplasm, extending in some cases to involve the whole cytoplasmic area (Figure 125). The remaining cytoplasm was condensed and arranged along the inner bacterial membrane. A smaller number were shrunken and stained more densely, resembling the organisms described in recovering PIA cases (Chapter IX). A few appeared characteristic of mucosalis, as described in typical PIA cases (Chapter III, Part A). In some of the organisms showing evidence of damage, the outer wavy membrane was possibly not continuous, although this may have been a sectioning artefact. Many of the bacteria were close to the luminal surface of the cells, and some were being released from damaged cells.

The increase in the number of cells in the lamina propria noted histologically was also evident ultrastructurally. Macrophages, lymphocytes and plasma cells were present, and also neutrophils and a variable number of eosinophils. The macrophages contained mucosalis in membrane-bound vesicles, and resembled those described in the lumina of the glands. Apoptotic bodies were also present in some macrophages.

The macrophages were seen within the epithelium and surrounding lamina propria of the adenomatous glands, and also in the lamina propria surrounding adjacent normal glands, although the epithelium of these latter contained no bacteria.

Immunofluorescence Results

Controls

The controls for this study were tissues from the normal pigs and typical PIA cases described in Chapter III, Part A. The results of staining with mucosalis anti-sera were presented at that time, and the results of staining with R.A.P. in Chapter IX.

PIA cases

When PIA cases were stained with 1248/72 2C2 mucosalis anti-serum, there was bright particulate fluorescence present within the apical cytoplasm of the affected epithelial cells. This was very bright and present in large quantities. Goblet cells in the deeper glands did not contain mucosalis antigen. There were also cells in the lumina of the glands, with particulate intracellular fluorescence and these most probably were the desquamated epithelial cells, neutrophils and macrophages demonstrated by electron microscopy. Free fluorescing vibrios were also present in the lumina of the glands. In the lamina propria there were cells, presumably macrophages, containing particulate intracellular fluorescence.

On staining parallel cryostat sections with 982/76 mucosalis anti-serum there was some particulate fluorescence in the apical cytoplasm of epithelial cells in some glands. The amount of fluorescence was much less than with 1248/72 2C2 mucosalis anti-serum. In the

lumina of some glands there were cells containing intracellular particulate fluorescence, and also in the lamina propria adjacent to the glands.

In parallel sections stained with R.A.P., there was a diffuse fluorescence in some of the epithelial cells, concentrated in the apical cytoplasm. Superimposed on this was a brighter, more particulate fluorescence corresponding to those areas which had fluoresced when stained with 1248/72 202 mucosalis anti-serum. There were also cells with intracellular particulate fluorescence in the lumina of the glands, and free fluorescing vibrios. This fluorescence again corresponded to that seen when staining was carried out using mucosalis anti-serum. In the lamina propria there were cells, adjacent to the glands which contained particulate intracellular fluorescence, probably macrophages and their position corresponded to that of the cells in the lamina propria, fluorescing when sections were stained with mucosalis anti-serum. There were also cells in the lamina propria, probably plasma cells, which contained diffuse intracytoplasmic fluorescence and which were usually present in large numbers. In areas with attempted villus formation and where adenomatous glands were "growing out", the demonstration of mucosalis by immunofluorescence corresponded to those areas in which vibrios were seen by electron microscopy and silver-staining. If these areas with adenomatous glands "growing out" were stained with R.A.P. then there was particulate fluorescence in the apical cytoplasm of the epithelial cells in the adenomatous glands. In the deeper histologically normal glands there was some fluorescence in the apical region of the epithelial cells, but this was less bright and diffuse rather than particulate.

Bacteriological Results

Fresh material and material examined twenty four to forty eight hours after death of the pig has not yielded mucosalis in large numbers. On one occasion from fresh material mucosalis was recovered in small numbers from the affected T.S.I. mucosa (Lawson, Rowland and Roberts, 1977a) (examination carried out by Dr. G.H.K. Lawson, Department of Veterinary Pathology, Edinburgh University Veterinary Field Station). The organisms recovered were serologically and biochemically identical to the neotype strain, 1248/72 202. The numbers of mucosalis recovered from this case were much lower than one would have expected from the number demonstrated to be present in modified Z.-N. stained impression smears of the mucosa, in silver-stained histological sections and by immunofluorescence and electron microscopy. Quantitative recovery recovery on M.B.G. indicated the presence of 2.4×10^2 viable organisms per gram, in contrast to the 10^7 organisms per gram expected from PIA cases.

DISCUSSION

This study confirms the underlying adenomatous change present in cases of PNE (Rowland and Rowntree, 1972; Rowland and Lawson, 1975; Rowland, Lawson and Roberts, 1976), and the demonstrable presence of mucosalis within the epithelial cells by silver-staining, immunofluorescence and electron microscopy. During the study, mucosalis was recovered from a case of PNE (Lawson, Rowland and Roberts, 1977a) and its isolation from similar material was reported from Australia (Love et al., 1977) and America (McAllister, personal communication). The organisms recovered from all three countries

resemble serologically and biochemically the neotype strain of mucosalis.

The results of the investigations reported here may help to explain the difficulty experienced in recovering mucosalis from cases of PHE (Lawson, Howland and Roberts, 1977a; Love, personal communication, 1977). The changes described have many features in common with the recovery phase of PIA. However, there are a number of important differences between the two conditions, and only these differences will be stressed in the discussion. Those factors relating to the recovery phase of PIA discussed in Chapter IX are in the main part also applicable to PHE. Immunofluorescent studies suggested that the bacteria were antibody coated, and they showed evidence ultrastructurally of degenerative change. They were present within membrane-bound vesicles in phagocytic cells in the lumina of glands and also within macrophages in the lamina propria. There was evidence both histologically and ultrastructurally of resolution of the lesions. The epithelium was severely damaged and there were more neutrophils and macrophages, predominantly neutrophils, present in the lumina of the glands than in recovering PIA cases.

In the PHE cases examined there were very large numbers of mucosalis in the epithelial cells, and possibly more than are seen in cases of PIA. Most of the organisms in the epithelial cells were showing degenerative changes with a loss of cytoplasmic staining and condensation of the cytoplasm on the inner bacterial membrane. This was in contrast to the usual changes seen in mucosalis in recovering PIA cases when the bacteria in the epithelial cells were densely osmiophilic and shrunken. The changes in mucosalis in the epithelial

cells in PHE resembled more closely those seen when the bacteria were present in phagolysosomes of neutrophils and macrophages in both PHE and recovering PIA cases. The reasons for these differences are unknown, one explanation being that the damage is antibody - mediated in both cases, but by a different class of antibody. The presence of mucosalis in large numbers in PHE may mean that the character of the immune response differs from that in pigs with PIA. The age of the pigs may have an effect on this response, both quantitatively and qualitatively; PHE is reported to occur in an older age group of animal.

The class(es) of antibody attached to the bacteria was not determined. Secretory IgA and secretory IgM are produced in plasma cells in the lamina propria of the intestine and pass through the intestinal crypt epithelial cells, where they are complexed with secretory component and passed onto the mucosal surface. It is conceivable that once specific mucosalis antibody is produced it may interact with mucosalis after entering the epithelial cell. Intracellular bacteria are usually considered to be safe from antibodies, but the transport of immunoglobulins across the intestinal epithelial cell may allow contact in this situation. Both IgA and IgM are able to bring about bacteriolysis, although the evidence for IgA is more controversial (Glynn, 1974). IgA was considered unable to activate complement, however it has now been shown to activate complement via the alternate pathway (Glynn, 1974). More recently, the Fc. fragment of IgA has been demonstrated to be able to activate complement via the classical pathway (Burritt et al., 1977).

The large numbers of neutrophils seen in the glands' lumina in PHL may be involved in the observed damage to both the bacteria and the intestinal epithelial cells. Neutrophils are attracted by antigen-antibody complexes, and in response to such complexes there is a release of lysosomal enzymes (Henson, 1971a; Hawkins, 1972). This occurs whether the immune complexes are susceptible to phagocytosis or not (Henson, 1971a and 1971b), and the process of enzyme release is not cytolytic for the donor cell (Henson, 1971b; Hawkins, 1972). Phagocytosis of bacteria has also been shown to stimulate enzyme release (Crowder, Martin and White, 1969). Release of lysosomal enzymes may be responsible for the degenerative changes in the bacteria and epithelial cells after the attraction of neutrophil polymorphs by antigen-antibody complexes involving mucosalis. The epithelial cell and bacterial damage is far greater in PHL than in recovering PLA cases, and there are larger numbers of neutrophil polymorphs in the former.

More recently the secretion of enzymes and lymphostimulatory molecules by macrophages has been recognised (Davies and Allison, 1976; Unanue, 1976; Unanue et al., 1976). Uptake of immune complexes is accompanied by a selective release of macrophage lysosomal enzymes from viable cells (Cardella, Davies and Allison, 1974). Selective release of acid hydrolases occurs in macrophages exposed to the products of stimulated T-lymphocytes, responding to specific antigen (Davies and Allison, 1976). Large numbers of macrophages were seen in this study and may also, like the neutrophils, have been involved in the release of enzymes, resulting in tissue and bacterial damage.

There was an increase in the epithelial cell loss from the mucosa compared to normal, which was evident both histologically and ultrastructurally. Where these cells were being shed, free red blood cells were also seen. This was almost certainly the source of haemorrhage; defects in the epithelium due to epithelial cell damage and loss allowing exposure of the underlying capillary bed, with subsequent damage and blood loss. Another possible contributing factor could have been damage to the vasculature by lysosomal enzymes from neutrophils and macrophages.

Associated with the degenerative changes in mucosalis, the immunofluorescent demonstration of antibody coating of mucosalis, and the failure to recover mucosalis in large numbers from the lesions, there was evidence that resolution of the adenomatous changes were beginning. As in the recovering PIA cases goblet cells were present in the deeper glands and in some areas there was attempted villus formation, and in others the adenomatous glands were "growing out". Apoptosis was observed in these PHE cases, both apoptotic cells and apoptotic bodies as described in Chapter IX were seen, which observation is consistent with an extensive remodelling of the epithelium.

Mucosalis was less commonly seen in membrane-bound vesicles in the epithelial cells in cases of PHE than in recovering PIA cases. This may be a reflection of the more severe damage to the epithelial cells in PHE, so that they are unable to remove mucosalis by "autophagocytosis", as discussed in Chapter IX. Occasionally, autophagolysosomes were seen containing mucosalis and degenerate cellular organelles.

Although their significance had not been realised a number of the changes seen in these cases had been commented on by other workers. O'Hara (1972) suggested that the histological lesions were of longer duration than the brief clinical illness suggested, and Pill (1971) described the almost complete resolution of the lesions on examination of pigs after three to four days of illness. Redman Chu and Hong (1971) described the presence of goblet cells in glands, but they do not indicate if these were only the deeper glands, or how many mucus-secreting cells were present. Clumps or syncytia of cells being lost from the mucosal surface in PHE was reported by Kelly and Cameron (1976) and this is in agreement with the increased epithelial cell loss in the cases examined here. Love, Love and Edwards (1977) reported that mucosalis showed evidence of degeneration in their cases, and that it was also present within cytoplasmic vacuoles in macrophages in deeper layers of the mucosa and sub-mucosa.

Further evidence that mucosalis in the intestinal mucosa in PHE cases is antibody coated has been obtained (Lawson, personal communication, 1977), by the extraction of the bacteria from the epithelial cells in two PHE cases (96/76 and 108/76). Formalised antigens were prepared and injected into rabbits in the manner used to produce hyperimmune mucosalis anti-serum (Lawson and Rowland, 1974). The rabbits failed to produce a response to the bacterial antigen from either one of these two pigs. From one of the pigs serologically typical mucosalis had been recovered (Lawson, Rowland and Roberts, 1977a), and both had shown specific epithelial fluorescence when stained with mucosalis anti-serum. The present author

examined an aliquot of the suspension of extracted bacteria from one of these animals ultrastructurally, and the organisms were identical to mucosalis. These findings are consistent with the report (Shearman, Parkin and McClelland, 1972) that secretory IgA can mask antigenic sites so that bacteria coated with secretory IgA appear to be non-antigenic in rabbits. Steel (1975) also showed that colostral IgA adsorbed to E. coli and inhibited the immune response to E. coli in rabbits and guinea pigs, by masking antigenic sites.

This study sheds some light on the pathogenesis of PHE, and helps to explain the difficulty previously experienced in recovering mucosalis from PHE cases. PHE appears to be a more acute recovery from a PIA case, with large numbers of neutrophils, extensive cell damage and haemorrhage, resulting in an acute clinical picture. The underlying changes and remodelling of the mucosa are, however, basically similar to those of the recovery phase of PIA. The large numbers of mucosalis present in the mucosa in PHE cases, and the age of the animals affected may be of significance in this condition, altering the immune response so that an acute clinical "recovery" occurs rather than the more gradual process described in Chapter IX. Further work will be necessary to elucidate the specific factors important in the particular immune responses involved in PHE.

CHAPTER XI

FINAL DISCUSSION

The major objectives of this study have been achieved and a greater understanding of the host-parasite relationship between mucosalis and the pig has resulted.

The field studies on naturally occurring cases of PIA, RI and NE demonstrated an underlying adenomatous change in the epithelium of all three conditions, and the presence of mucosalis in the epithelial cells was established. The unique relationship between mucosalis and the epithelial cell, with the bacteria free in the cytoplasm and causing no inflammatory or degenerative change confirmed the previous descriptions (Rowland, Lawson and Maxwell, 1973; Rowland and Lawson, 1974). The epithelial cell infiltration seen in some PIA cases with superficial mucosal damage has not previously been reported, although metastases to the mesenteric lymph nodes were documented by Emsbo (1951) and Nielsen (1971).

During the work a serologically distinct variant of mucosalis was isolated (Roberts, Lawson and Rowland, 1977). Since then a further serologically distinct variant of mucosalis has been recovered by Dr. G.H.K. Lawson, Department of Veterinary Pathology, Edinburgh University Veterinary Field Station, and these are now referred to as serotypes A, B and C. Serotype A includes those mucosalis strains which share surface antigens with the neotype strain, 1248/72 2C2; serotypes B and C are used for those strains resembling 982/76 and 512 /77 respectively. There is no cross reaction between the three serotypes in slide and tube agglutination tests using "OH" antigens.

The significance of the serotypic variation is unknown. Both serotypes A and B have been demonstrated free in the cytoplasm of host epithelial cells. For serotype C, this has not yet been shown - the recovery of the isolate of serotype C was made from the faeces of a gilt with clinical signs typical of PHE. Antigenic variation within a particular serotype has also been demonstrated (Lawson, Rowland and Roberts, 1977b). This is seen as differences in surface antigenic factors, although the isolates still share major surface components. Such differences are seen between isolates from different herds, isolates from one herd over a period of time, isolates from different regions of the U.K., and between isolates from the U.K. and Sweden. Antigenic variation has been described for Vibrio cholerae in the intestines of gnotobiotic mice (Miller et al., 1972), Streptococcus mutans in the intestinal tract of gnotobiotic rats (Bratthall and Gibbons, 1975b) and campylobacters in the male and female genital tracts (Schurig et al., 1973; Corbeil et al., 1975; Bier et al., 1977). With C. fetus subsp. intestinalis infection of virgin heifers some of the isolates recovered during the experiment were serologically quite distinct from the inoculating strain. These alterations are thought to be due to selection pressure brought about by antibodies directed against the organisms involved.

Mucosalis was also recovered from piglets in a "three week scours" problem, which occurred on a farm where PIA was known to be concurrently an endemic disease. This was the first report of the recovery of mucosalis from the intestinal tract of naturally diseased pigs, without an underlying adenomatous proliferation in the mucosa. The results of that work indicated that the role of mucosalis should

be considered in outbreaks of the poorly-defined condition referred to as "three week scours" (Mouwen, 1972).

A study of PIA in a closed M.D. herd was also undertaken and 2.5% of the throughput of the herd were confirmed as having PIA, and at least a further 2.5% were probably also affected. These cases would mostly, if not entirely, have gone unnoticed, but for the fact that weekly weights for the pigs after weaning were available, and were analysed to select pigs for examination. This was the herd in which the outbreak of PIA and PHE described by Rowland and Rowntree (1972) occurred in 1966. Since the original outbreak it was considered that the herd had been free of PIA and mucosalis infection. The results of the investigation reported in Chapter III, Part C shed some doubt on that assumption.

A series of infectivity experiments (Chapters IV, V and VI) were undertaken to obtain information about the host-parasite relationship between the pig and mucosalis. Pigs were successfully infected with mucosalis, but only at a low level. The neonatal piglet was found to be more susceptible than older animals to oral infection, and mucosalis could be recovered up to forty days from both the intestinal mucosa and the oral cavity. Whether dosing was carried out before or after suckling did not appear to alter the susceptibility to infection with mucosalis, but it did influence the site(s) at which infection became established. The post-weaned piglets were found to be more resistant to mucosalis infection under the circumstances of the experiments. Their susceptibility was increased by the administration of the anticholinergic agent, benzetimide, to reduce peristalsis. In a more detailed study of the infection of

neonatal piglets with mucosalis with special reference to the oral cavity it was shown that mucosalis could establish in the mouths of a number of piglets in a litter and be maintained in that site within the litter for up to eight weeks. Not all of the piglets in the litter yielded mucosalis each week, but at least some were positive. In the experiment just described coincident with the disappearance of mucosalis from the mouths of these piglets other catalase negative, serologically distinct campylobacters were recovered, this could indicate a change in the surface antigens of the infecting strain, although other explanations are possible.

This susceptibility of the neonate to mucosalis infection is of interest with respect to the source of mucosalis infection. In the litter of sow D (Chapter IV) one piglet (87/75) yielded mucosalis isolates of both serotypes A and B, from the M.S.I. mucosa at necropsy. The piglet had been dosed at birth with mucosalis of serotype A, but had no known contact with mucosalis of serotype B. The only animal, other than its littermates, which this piglet had contact with was the sow and it is possible that infection was acquired from this source. In sow D, catalase negative campylobacters, which did not react in slide agglutination tests with 1248/72 2C2 mucosalis anti-sera (N.A.C.s) were recovered from the oral cavity six days before farrowing. At six days of age, two out of four of her piglets sampled were positive for N.A.C.s, and it is tempting to suggest that these campylobacters were acquired from the sow. The acquisition of members of the oral microbial flora by neonates from the dam is becoming widely accepted (Chapter VI). In Tyzzer's disease in the foal, the causative organism Bacillus piliformis is picked up from the faeces of carrier mares

by coprophagy (Swerczek, 1977). Further work will be necessary to clarify many of these points, including a thorough examination of the campylobacters involved.

No carrier state for mucosalis in the pig has been described to date. Mucosalis has been recovered from the oral cavities of piglets (Lawson, Rowland and Roberts, 1975), but not from adult pigs, although only a small number of adult animals have been examined. Other microaerophilic campylobacters have been recovered from the oral cavities of adult pigs, so it is possible that mucosalis may also occur in this site. Vaginal swabs were also taken from sows during this study (Chapter VI), and although other campylobacters were recovered, mucosalis was not isolated. Microaerophilic vaginal vibrios have been described in the human (Moore, 1954) and C. fetus subsp. venerealis, C. fetus subsp. intestinalis and C. sputorum subsp. bubulus inhabit the genital tract of cattle or sheep. It would be of value to determine if a carrier state for mucosalis occurs, and the site of any such infection. Transmission of campylobacters, including mucosalis, from the sow to her litter, in view of the susceptibility of neonatal piglets to mucosalis infection, would seem to merit further investigation.

In C. fetus subsp. venerealis infection of cattle the bull is the carrier, and the susceptibility and the persistence of infection may increase with age, this susceptibility possibly being related to an increase in the number and size of crypts in the epithelium of the penis (Samuelson and Winter, 1966), although more recent evidence suggests that persistence of the organism and development of the carrier state is not age related (Bier et al., 1977). With C. fetus subsp.

intestinalis in sheep, after abortion the organism can persist in the gall bladder or intestines of some sheep (Jensen, 1974). C. fetus subsp. intestinalis has also been recovered from captive magpies 213 days after the birds had ingested cultures of the microorganism, Other magpies caged with the infected birds also became infected, and the isolates recovered from the birds were pathogenic for sheep (Meinershagen, et al., 1965). Campylobacters have also been recovered from wild bank voles (Ferne and Healing, 1976) and laboratory rodents (Ferne and Park, 1977). The role that wild birds or rodents play in transmission of mucosalis remains to be investigated.

The transmission experiments (Chapters VII and VIII) were also successful and further emphasised that at our present state of knowledge the neonatal piglet is probably the most useful experimental animal. Using homogenised adenomatous mucosa from a clinical case, PIA was successfully transmitted to a litter of piglets, which were dosed orally in the neonatal period. Benzetimide was also used to reduce peristalsis in the dosed animals. When the same mucosa was used after storage at -80°C , there was a greatly reduced success rate. In the post-weaned pig, transmission of PIA using adenomatous mucosa and benzetimide administration, which had been shown to increase the susceptibility to mucosalis infection, was not successful.

The piglets in the successful transmission experiment were killed over a period, including the stage at which developed PIA and NE lesions were present and this allowed the examination of pre-adenomatous cases and cases in the recovery phase of PIA. The recovery phase of PIA was also recognised in the field studies and

both naturally occurring and experimental cases were examined in detail. Although clinical recovery of pigs affected with PIA has been reported, this was the first study of the cellular events in this recovery, with the elimination of mucosalis and return of the epithelium to normal.

PHE cases were also examined and a better understanding of the pathogenesis of the condition and the difficulty in recovering mucosalis from such cases (Lawson, Rowland and Roberts, 1977a) was reached. The PHE cases had many features in common with the recovering PIA cases, and the impression gained was that it was a more acute recovery of a PIA case, with extensive tissue damage and resultant haemorrhage. Affected animals either die acutely with a haemorrhagic anaemia and shock, or recover quickly and uneventfully.

It was suggested that one possible factor in the greater susceptibility of neonatal pigs to mucosalis infection than post-weaned pigs was the longer cell cycle time in the epithelium of the former. Cell kinetics in the intestinal epithelium with special reference to mucosalis infection and PIA would appear to be a potentially important area warranting further study and the results might shed light on the pathogenesis of PIA and on the predisposing factors and susceptibility to mucosalis infection. For an intracellular parasite such as mucosalis, in a tissue with a relatively rapid turnover it is possible that the cell kinetics would be intimately related to the persistence of mucosalis infection. The use of gnotobiotic animals might prove to be of value in this context, since germ-free animals have an intestinal epithelial cell turnover time double that

of conventional animals, the lifespan of individual epithelial cells being correspondingly increased (Abrams, Bauer and Sprinz, 1963). Starvation results in a reduction in D.N.A. synthesis, cell proliferation and migration in the intestinal epithelium (Brown, Levine and Lipkin, 1963) and withholding food might be of use in experimental studies. One could also argue that the converse of this might similarly be true, and that when the cell proliferation and migration rates are increased the availability of more immature intestinal epithelial cells and at higher levels in the mucosa may influence the infection rate. It is difficult to imagine parasitism by mucosalis causing dedifferentiation of the previously mature epithelial cell, even though evidence at present suggests that the presence of the organism is linked with the failure of intestinal epithelial cells to differentiate in PIA. For this reason if there is an increased proliferation of the intestinal epithelium, with more immature cells migrating towards the surface and onto the villi the chances of establishing mucosalis in the epithelium might be enhanced. In certain intestinal infections where measurements have been made, there are increased epithelial cell proliferation and migration rates, e.g., in TGE in piglets (Thake, Moon and Lambert, 1973). This results in a greater number of immature epithelial cells being present at higher levels on the villus (Kerzner et al., 1977). In this context the role of the apparently widely distributed rotavirus in causing epithelial damage and predisposing to mucosalis infection would seem to warrant further study. The susceptibility to mucosalis infection and cell kinetics is likely to be complex and delicately

balanced. For example, after weaning food intake is often reduced, and this may influence cell kinetics resulting in a reduction in the epithelial cell turnover. A "physiological inflammation", with resulting villous atrophy and crypt hyperplasia, has been described after weaning and although cell kinetics studies have not been undertaken the impression is that there is an increased epithelial cell turnover rate at that time. The interplay of many factors and their effect on epithelial cell turnover could be critical in the development of the disease.

The application of cell kinetics studies to PIA may also prove of wider significance in the study of gastro-intestinal neoplasia. Considerable evidence now indicates that changes in the proliferative pattern of colonic epithelium may precede the development of polyps or carcinoma of the colon (Eastwood, 1977). Normally cells which migrate from the proliferative zone towards the mucosal surface undergo changes in the pattern of their nucleic acid metabolic enzymes, which are consistent with their inability to synthesise D.N.A. (Lipkin, 1973). Lipkin has devised a theoretical model to explain the changes in proliferation kinetics which may occur during the development of colonic neoplasms (Lipkin, 1974). In the first stage of development of a proliferative lesion cells migrate up beyond the usual limits of the proliferative zone, but do not repress D.N.A. synthesis and retain the ability to proliferate. The overall process of proliferation, migration, and extrusion of cells remains normal, without net retention of cells within the mucosa - the proliferative zone has in fact expanded to include the upper crypt

and surface epithelium. In the second stage cell renewal kinetics become abnormal, with net retention and accumulation of cells within the mucosa. The cessation of cell loss and the persistence of abnormal proliferation kinetics results in polyp formation. PIA could prove useful as a model to study the factors involved in these alterations in proliferative activity and cell loss, as in PIA there are mitoses present at all levels of the epithelium, and histologically the condition resembles a neoplasm.

The studies reported in this thesis have involved in vivo work, and in the future application of in vitro techniques would seem indicated to provide answers to important problems relating to mucosalis and the parasitised cell. For mucosal pathogens, attachment to the epithelium has been shown to be of importance (Smith, 1977), and has been studied extensively for V. cholerae (Jones, 1975; Nelson, Clements and Finkelstein, 1976), and E. coli (Jones and Rutter, 1972; Wilson and Hohmann, 1974) using a variety of methods. It would seem likely that there is an interaction between mucosalis and the surface of the epithelial cell before entry/uptake and in vitro studies might help to provide a greater understanding of this facet of mucosalis infection. Rickettsia (Anderson et al., 1965) and bacteria (Fitzgerald, Miller and Sykes, 1975; Brodeur et al., 1977) have been studied using tissue cultures, and initial work with mucosalis in a continuous kidney cell tissue culture system has proved promising (Roberts, unpublished results). As well as providing insight into attachment and entry this system would also provide a more controllable environment than the intestinal tract, in which to

study the biochemistry of the host-parasite interaction and factors important in successful infection of cells. Isolated intestinal epithelial cells have been used in work with E. coli (Wilson and Hohmann, 1974), and with both V. cholerae (Jones, Abrams and Freter, 1976; Jones and Freter, 1976) and E. coli (Sellwood et al., 1975) interactions with isolated brush border membranes have been studied. Receptor specificity is important in such interactions and in this context the K88 antigen has been described as the adhesive factor in some porcine enteropathogenic E. coli serotypes (Jones and Rutter, 1972), and K99 plays a similar role in some calf enteropathogenic E. coli serotypes. Increasing use is being made of organ cultures in in vitro work of this type and slices of rabbit ileum were used in a study to define the nature of the interaction of V. cholerae with intact mucosal surfaces (Freter and Jones, 1976). Use of these methods, isolated intestinal epithelial cells or brush borders and organ culture should provide information on the initial interaction between mucosalis and the host cell. Scanning electron microscopy could also be useful in studies of this type and has to date proved useful in work with V. cholerae (Nelson, Clements and Finkelstein, 1976) and in studying the entry of mycoplasma (Jones, Minick and Yang, 1977) and toxoplasma (Aikawa et al., 1977) into tissue culture cells. A variation on the organ culture technique would be to use ligated loops of intestine.

The entry or uptake of mucosalis is particularly of interest since mucosalis is free within the host cell cytoplasm in contrast to the situation with the majority of other intracellular intestinal bacteria (Staley, Jones and Corley, 1969b; Takeuchi, 1971). If

uptake is by phagocytosis then presumably mucosalis is contained within a membrane-bound phagosome from which it escapes. Uptake by phagocytosis and subsequent escape from the phagosome to replicate free in the cytoplasm has been reported for Rickettsia mooseri (Andrese and Wisseman, 1971) and Trypanosoma cruzi (Nogueira and Cohn, 1976). Direct penetration as a method of entry into non-phagocytic mammalian cells has been described for T. cruzi (Tanowitz et al., 1975). Whether or not one of these is the method by which mucosalis comes to lie free within the host cell cytoplasm has still to be elucidated. A tissue culture system would seem to be the method most likely to provide answers to this problem, the chance encounter at an ultra-structural level of this process in the live animal being very small.

Epidemiological studies of the presence of mucosalis in the pig population, or the incidence of PIA and related proliferative enteropathies have to date not been undertaken. As discussed in the Introduction (Chapter I), PIA is worldwide in occurrence, but without a reliable diagnostic test in the live animal such work would be difficult and in the future efforts should be made to develop such a procedure. For this purpose serology would seem to be worth further investigation, serological tests have been described for C. fetus infection in man (Bokkenheuser, 1972) and animals (Carter, 1973). Carriers of mucosalis have already been discussed, and a number of other points relating to epidemiology may be of relevance. The susceptibility of the neonate to infection and the possible acquisition of mucosalis from the dam may be important. If piglets became infected neonatally in this manner, either in the intestinal mucosa

or in the mouth, they could then provide a source of infection for other uninfected piglets at weaning. These latter may then go on to develop PIA, which would be consistent with the natural occurrence of PIA in the post-weaned pig.

Another facet of significance in the epidemiology is the question of individual, strain or breed susceptibility. For E. coli in piglets a genetic susceptibility to E. coli infection has been described, which is related to the presence or absence of receptors on the intestinal epithelial cell for the K88 antigen (Sellwood, et al., 1975). In the neonatal piglet infectivity experiments none of the piglets of one sow (C) became infected with mucosalis after exposure, in contrast to those of the other sows. One possible explanation for this would be an inherent resistance of the type described for E. coli. Interestingly a retarded growth syndrome has been described in Hampshire piglets (Danion, Squiers and Tucker, 1969; King, 1975). No cause for this poor growth was found and Danion et al., reported that not only did their pigs fail to grow well between thirty five and fifty six days, but some actually lost weight. The herd from which the pigs studied by King were derived is herd A, and PIA is endemic in this unit. Furthermore, some of the pigs used in these experiments included Hampshires in their breeding, and they have proved susceptible to mucosalis infection. Elucidation of the susceptibility of individuals, strain or breed would facilitate the selection of experimental animals.

The immune response to mucosalis infection warrants investigation. It is accepted that pigs affected with PIA recover, and

this recovery has been studied in detail (Chapter IX). PHE resembled this recovery process, but was more acute. A study of the immune response to mucosalis in the two conditions might help to explain the differences between them. The lack of response to mucosalis in the intestinal epithelial cell before recovery is intriguing, and it is not known at present why the host does not initially recognise mucosalis as foreign. The immune responses of specific-pathogen free and gnotobiotic mice to antigens of indigenous microorganisms are less than to non-indigenous microorganisms, and in one case no immune response was detectable to an indigenous strain of *Bacteroides* injected parenterally (Foo and Lee, 1972). Certain oral microorganisms are able to adsorb blood group reactive substances from saliva or produce them de novo, and in this manner may evade the host immune mechanisms (Gibbons and Qureshi, 1976). The significance of these in mucosalis infection remains to be determined. That pigs do mount a serological response to mucosalis (Lawson and Rowland, 1974) and recover from mucosalis infection (Chapter IX) suggests that vaccination may in the future be possible to prevent mucosalis infection and development of PIA. For C. fetus subsp. venerealis in cattle vaccination has been found useful in the cure and prevention of vibriosis (Campylobacterosis) in both males (Clark, Dufty and Monsborough, 1968a; Bouters et al., 1973) and females (Clark, Dufty and Monsborough, 1968b; Schurig et al., 1975) and also for C. fetus subsp. intestinalis in sheep (Storz et al., 1966).

The work presented in this thesis considerably extends our knowledge of PIA, and the host-parasite interaction between mucosalis

and the pig. However, many facets of the disease remain unstudied and possible areas in which future research might be rewarding have been outlined. The further development of the neonatal piglet model for transmission would be of considerable value to study further the pathogenesis, and the value of treatment and vaccination. The more detailed aspects of the host-parasite interaction such as attachment, uptake/entry and the initial ultrastructural and biochemical cellular changes after infection would be better studied using in vitro techniques. Throughout this study it has emerged that PIA, and the other proliferative enteropathies, EIEC and PHE are more than just biological curiosities and are, in fact, important conditions, contributing possibly significantly, to loss in the pig industry.

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APPENDIX IINVESTIGATION OF INCREASED INHIBITION OF MUCOSALIS BY N.B.G.

One batch of N.B.G. plates were observed to be excessively inhibitory for mucosalis, since mucosalis was isolated from a number of mucosal samples on B.A., but not on N.B.G. This was confirmed by carrying out duplicate counts of mucosalis on the N.B.G. and on B.A. plates using pure cultures of mucosalis (Miles and Misra, 1938). Two strains of mucosalis were used, 106/75 and 1248/72 2C2. Strain 106/75 was found to be the most sensitive to the inhibitory effect. Attempts were made to determine the cause for this, but were not successful. The problem however disappeared and retrospectively this was correlated with media made from a new batch of base. Subsequently on each occasion that N.B.G. plates to be used for the recovery of mucosalis from experimental animals were poured, a number of these plates were tested with mucosalis strains 106/75 and 1248/72. This was to ensure that the plates were not excessively inhibitory for mucosalis.

A number of months later this problem recurred and attempts were again made to find the cause, initially without success. It was then realised that the batch of base was almost finished and plates were poured using either a freshly opened batch of base or the old base. The latter was excessively inhibitory, while the former allowed growth of mucosalis at the same level as non-inhibitory media, or did not cause a reduction greater than one \log_{10} .

The routine checking of N.B.G. plates was carried out as follows:-

Mucosalis cultures (106/75 or 1248/72) were grown on C.S.A./tryptose phosphate slopes for 24 hours, under microaerophilic conditions, and checked for purity. A 0.02 ml drop of culture was added to 9 mls 0.1M pH 7.2 P.B.S. and then three tenfold dilutions made. 0.02 ml drops of these four dilutions were then used to inoculate the N.B.G. plates by the method of Miles and Misra (1938). Non-inhibitory control media (B.A. or C.B.A.) was also inoculated. If the count of mucosalis showed a greater than tenfold reduction on the N.B.G., as compared to the non-inhibitory media, it was considered to be excessively inhibitory.

Subsequently it was found that the old basal media would not support the growth of mucosalis, even in the absence of inhibitory substances (Novobiocin or brilliant green), and it was concluded that some change had taken place in the base. Possibly this could be a labile essential nutrient and clearly if so it was not provided adequately by the inclusion of blood in the media at the level of 5%.

Porcine intestinal adenomatosis associated with serologically distinct *Campylobacter sputorum* subspecies *mucosalis*

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A variant of *Campylobacter sputorum* subspecies *mucosalis* which is serologically distinct from all strains previously examined is described. It has been isolated from cases of porcine intestinal adenomatosis and necrotic enteritis and in all other characteristics conforms to the description of *mucosalis*.

PORCINE INTESTINAL ADENOMATOSIS (PIA) is a proliferative enteropathy of the pig in which there is a constant association with an intracellular bacterium (Rowland *et al* 1973; Rowland and Lawson 1974). The bacterium is a vibrio, which has been characterised and the accepted name is *Campylobacter sputorum* subspecies *mucosalis* (Lawson *et al* 1975). The organisms lie free within the apical cytoplasm of affected intestinal epithelial cells (Rowland and Lawson 1974) and a similar relationship has been described in Sweden (Martinsson *et al* 1974; Jönsson and Martinsson 1976). *Mucosalis* isolated by the latter workers (Gunnarsson *et al* 1976) has been examined in this laboratory and is serologically closely related to those strains already described (Lawson and Rowland 1974; Lawson *et al* 1976). Lawson and Rowland (1974) found that an antiserum prepared against one of the first strains of *mucosalis* isolated from a case of PIA reacted with all subsequent *mucosalis* strains examined. *Mucosalis* strains recovered from other porcine proliferative enteropathies, regional ileitis (RI) and necrotic enteritis (NE), are also closely related serologically to the *mucosalis* strains from PIA cases (Lawson *et al* 1976).

In this report, we wish to describe strains of *mucosalis* isolated from cases of PIA and NE which are serologically distinct from those strains of *mucosalis* already described.

Materials and methods

The pigs in this study were obtained from two farms. The pig from herd B was submitted because of wasting to the point of emaciation. Two of the pigs from herd K were received dead for post mortem examination; the third was submitted alive at our request.

The pathological and bacteriological procedures have been described previously (Rowland and Lawson 1974; Lawson and Rowland 1974). In addition, impression smears were prepared from the washed mucosa and stained by the modified Ziehl Nielsen technique (Love *et al* 1977) and histological sections were stained by two silver techniques (Levaditi and Manouelian 1906; Young 1969).

Results

History and clinical signs

The 11-week-old pig from herd B was in extremely

poor condition and had wasted to the point of emaciation. This herd has a history of wasting pigs due to PIA, from which *mucosalis* has been isolated consistently. *Mucosalis* isolated from this herd had up to this time behaved typically (Lawson *et al* 1975; Lawson *et al* 1976).

The other three pigs were from a closed experimental herd (K), recently established from a neighbouring closed pure Large White minimal disease herd. Two of the pigs were 12 weeks old and were presented for post mortem examination as sudden deaths in already poor pigs. The third pig was 12 weeks old and was presented live at our request. It was in poor bodily condition but not apparently diarrhoeic.

Pathology

The pig from herd B had lesions of PIA (Rowland and Rowntree 1972) involving the large bowel. The two dead pigs from herd K had lesions of NE (Rowland and Lawson 1975), while the third animal had lesions in the large intestine of PIA. The histological changes confirmed that the gross lesions were typical and Levaditi and Manouelian's (1906) and Young's (1969) stained sections showed bacteria with vibrio morphology in the apical cytoplasm of cells in the affected glands.

Bacteriological findings

Impression smears stained by the modified Ziehl Nielsen technique showed the presence of acid fast intracellular vibrios within the intestinal epithelial cells. Occasionally, clumps of free acid fast vibrios were also seen. The pig (982/76) from herd B yielded 4.96×10^6 colonies per g of tissue which had the morphological, cultural and biochemical characteristics of *mucosalis*. Uncharacteristically, however, subcultures from these colonies did not react with our standard antisera (253/72 [NCTC 11,000]) in slide agglutination tests. In tube agglutination tests, 'OH' antigens prepared from representative colonies failed to react with 253/72 'O' or 'OH' antisera at dilutions of 1/20 (homologous titres of the antisera 253/72 $\geq 1/1280$). An antiserum prepared against one strain from this pig (982/76) reacted with the homologous antigen in both slide and tube agglutination tests but not with the other strains which react with 253/72 antisera.

The isolates from the pigs from herd K were similar to those from pig 982/76 and conformed to the morphological and biochemical description of *mucosalis*. These isolates reacted with the antiserum prepared against the *mucosalis* strain from pig 982/76 but not with the antisera against other *mucosalis* strains.

Immunofluorescent findings

Tissue from pig 982/76 was not available for immunofluorescent staining. Cryostat sections of the large intestine from pig 1405/76 (herd K) were stained using the antiserum prepared against the *mucosalis* strain from pig 982/76. Positive particulate fluorescence was detected in the apical cytoplasm of cells in affected glands but not in the cells of histologically normal glands. Parallel sections stained using our standard *mucosalis* antiserum prepared against 253/72 were negative. Similarly, immunofluorescent staining of tissue, from which serologically typical *mucosalis* had been isolated, with 982/76 antiserum did not give positive fluorescence, while parallel sections stained with 253/72 antiserum did show positive fluorescence.

Electron microscopic examination

Electron microscopic examination of tissue from pig 1405/76 confirmed the presence of intracellular bacteria in the cytoplasm of the intestinal epithelial cells in affected tissue. These bacteria were free within the cytoplasm and did not appear to be surrounded by host/cell membranes, thereby conforming to the host/cell/bacterial relationship already described for *mucosalis* (Rowland and Lawson 1974). The affected epithelial cells ultrastructurally resembled immature crypt cells (Toner *et al* 1971).

Discussion

The bacteria isolated from these cases appear to conform to the description of *mucosalis* but are serologically distinct from those strains of *mucosalis* already examined (Lawson *et al* 1975; Lawson *et al* 1976; Lawson *et al* 1977). Isolates of *mucosalis* from a number of herds, the same herd over a number of years, different regions in the UK and from the UK and Sweden are serologically closely related, although detectable differences are present (Lawson *et al* 1977). The *mucosalis* strains described in this report are not serologically related to those other *mucosalis* strains examined and there does not appear to be any cross reaction between the two groups with respect to the superficial antigens. The relationship between these two serologically distinct groups of *mucosalis* is as yet unclear. Variation in the superficial antigens in the *mucosalis* strains isolated from pigs in one herd over a number of years has been described (Lawson *et al* 1977) but these strains are still closely related to each other. Similar antigenic variation has been described for *Campylobacter fetus* (Schurig *et al* 1973; Corbeil *et al* 1975). With experimental infection of virgin heifers with *C. fetus* subsp. *intestinalis*, an antigenic drift is described and some of the isolates recovered during the experiment do not react at all with an antiserum prepared against the infecting strain. The possibility exists that antigenic variation in this group of organisms may be minor with the strains remaining closely related or major when serological cross reaction is no longer detectable.

A further pig, killed at the same time as 982/76 and

from the same farm, showed changes of PIA and bacteriological examination yielded a *mucosalis* serologically typical of those already described but distinct from the *mucosalis* isolated from 982/76. Furthermore, isolates of the two serologically distinct variants of *mucosalis* have been isolated from the same intestinal mucosal sample in one pig.

In the meantime, we continue to retain the name *mucosalis* to identify those organisms which may be isolated from adenomatous tissue or serologically and biochemically identical organisms isolated from other sites. To encompass the antigens of the subspecies now requires two antisera prepared against the surface antigens of strains 253/72 and 982/76 rather than a single antiserum as previously.

Acknowledgments

This work was supported by a grant from the Wellcome Trust while one of us (L. R.) was in receipt of a Wellcome Trust Scholarship.

Received for publication March 18, 1977

Accepted April 4, 1977

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Experimental reproduction of porcine intestinal adenomatosis and necrotic enteritis

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Vet. Rec. (1977), 100, 12-13

PORCINE intestinal adenomatosis (PIA) is an enteropathy of the pig which resembles a transient neoplasm, the lesion consisting of immature proliferating epithelial cells. The duration and epidemiology of the condition are, however, those of an infectious disease. Bacteria were first described in the adenomatous tissue by Rowland and others (1973) and Rowland and Lawson (1974). Subsequently, a similar cell/bacterial relationship has been described in Sweden (Martinsson and others 1974, Jönsson and Martinsson 1976). The bacterium involved has been isolated and named *Campylobacter sputorum* subspecies *mucosalis* (Lawson and others, 1975). We wish to report here the experimental production of PIA and necrotic enteritis (NE).

Sows of conventional health status were purchased from one farm, farrowed in isolation and the piglets exposed to a variety of treatments in the neonatal period. The sows and litters were maintained in isolation, creep introduced from nine days and weaning carried out at three weeks.

Nine 18-hour-old piglets from one litter were dosed intragastrically with 1 g of chalk powder in 5 ml distilled water, benzetimide (Marsboom and others 1973) at 0.25 mg/kg body weight and 10^{10} viable *C. sputorum* ss *mucosalis*. In addition, each piglet received 5 ml of homogenised adenomatous mucosa. This last was derived from two histologically confirmed clinical cases of PIA and was prepared as follows:

Immediately after euthanasia of the pigs, the affected mucosa was washed, stripped and weighed, mixed with an equal weight/volume of tryptose phosphate broth and homogenised. The mucosal suspension was used for dosing within three hours of death and contained 6.8×10^7 *C. sputorum* ss *mucosalis* per ml of homogenate.

Control piglets, born to sows from the same source and treated up to farrowing in an identical manner, were dosed in the neonatal period with suspension of *mucosalis* only.

The piglets dosed with tissue gained weight poorly, this becoming clinically apparent from three weeks and in addition their appetites appeared capricious. The animals were killed at 24, 38, 51, 53, 59 (two), 65 (two) and 66 days of age. Increasing numbers of *mucosalis* were isolated from the mucosa of piglets killed up to 53 days of age, reaching numbers equivalent to those obtained in naturally occurring PIA cases. The three piglets killed at 53 and 59 (two) days had lesions of PIA as previously described (Rowland and Rowntree 1972, Rowland and Lawson 1974). The pig killed on day 51 had lesions of NE (Rowland and Lawson 1975).

The pigs killed at 65 (two) and 66 days had latterly shown improved weight gains. *Mucosalis* was not recovered from these last three animals and there was an absence of the clear cut proliferative lesions seen in the mucosa of pigs killed earlier in the experiment.

Bacteria resembling *mucosalis* were demonstrated in Levaditi stained sections or by electron microscopic examination in the apical cytoplasm of the proliferating epithelial cells. Immunofluorescence using hyperimmune *mucosalis* antiserum (Lawson and Rowland 1974) also confirmed the presence of the organisms in the apical cytoplasm of the altered epithelial cells.

The control piglets were killed over a similar period of time, and although small numbers of *mucosalis* were recovered from some animals, gross and histological changes as described in this report were not seen.

This preliminary communication provides information on the incubation period of the clinical disease and its duration. It also confirms the intimate association between the bacterium, *C. sputorum* ss *mucosalis*, and the epithelial proliferation of the mucosa. Although the circumstances necessary for the production of the disease are by no means clear, this experiment provides additional evidence of the infectious nature of the condition and of the relationship between PIA and NE, which has already been postulated.

Acknowledgements.—This work was supported by a grant from the Wellcome Trust and one of us (L. Roberts) is in receipt of a Wellcome Trust Scholarship. We also wish to acknowledge a gift of benzetimide from Janssen Pharmaceutica.

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